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(54) Title: DISEASE ASSOCIATED HUMAN AUTOANTIBODIES SPECIFIC FOR HUMAN THYROID PEROXIDASE

(57) Abstract

     Disease associated human autoantibodies specific for human thyroid peroxidase are disclosed. Novel organ-specific (TPO)
human autoantibodies are disclosed which have been cloned, allowing definition of the autoantibody repertoire and the autoanti-
genic domains, encompassing a restricted immunodominant region on TPO recognized by patients with autoimmune thyroid dis-
ease. The novel compositions of the invention, their diagnostic and therapeutic applications, are, inter alia disclosed.
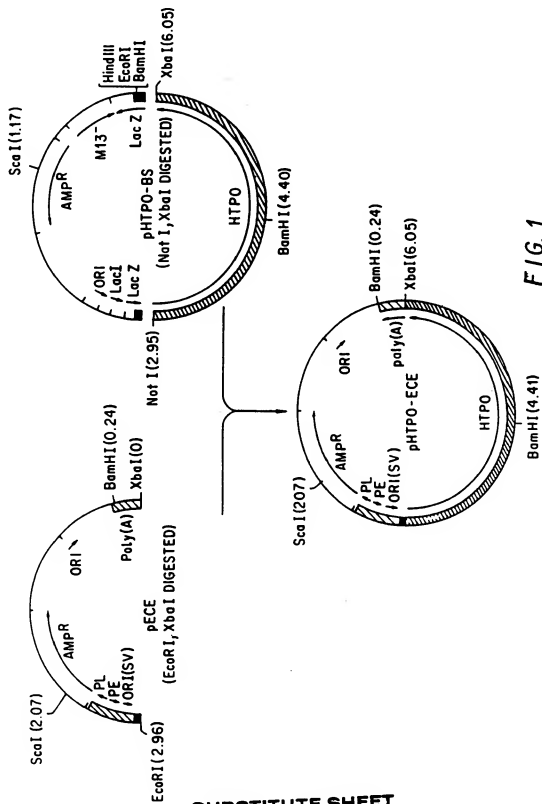
*FIG. 1*

## TITLE OF THE INVENTION

## DISEASE ASSOCIATED HUMAN AUTOANTIBODIES SPECIFIC FOR HUMAN THYROID PEROXIDASE

## BACKGROUND OF THE INVENTION

### Cross-Reference to Related Applications

This application is a continuation-in-part of International Application No. PCT/US92/06283, filed July 30, 1992, United States Patent Application Serial No. 07/750,579, filed August 28, 1991, and United States Patent Application Serial No. 07/738,040, filed July 30, 1991, which is a continuation-in part of United States Patent Application Serial No. 07/559,955, filed July 31, 1990, which is a continuation-in-part of United States Patent Application Serial No. 07/472,070, filed January 30, 1990, which is a continuation-in-part of United States Patent Application Serial No. 07/388,044, filed July 31, 1989.

### Background of the Invention

Part of the work leading to this invention was made with U.S. Government funds. The U.S. government has certain rights in this invention.

### Field of the Invention

The present invention relates to the fields of molecular biology and immunology. More particularly, the invention relates to the production of recombinant human thyroid peroxidase in non-thyroidal eukaryotic cells. The invention is further related to methods of using recombinant human thyroid peroxidase, and, in particular, to methods of using recombinant human thyroid peroxidase in diagnosis and treatment of immune disorders such as Hashimoto's thyroiditis.

## Brief Description of the Related Art

Hashimoto's thyroiditis is the most common autoimmune endocrinopathy, affecting, at least subclinically, up to 15%
5    of the adult female population (Volpe, R., In _Werner's The Thyroid_, 5th Edition (Ingbar, S.H., _et al._, Eds.), J.B. Lippincott Co., Philadelphia, pp. 1266-1291 (1986); Gordin, A., _et al._, _Acta Endocrinol._ _90_:33-42 (1979)).  Antibodies against a number of thyroid antigens are present in the sera
10   of these patients, including thyroglobulin and the thyroid "microsomal" antigen (Doniach, D., _et al._, _Clin. Endocrinol. Metab._ _8_:63-80 (1979); Weetman, A.P., _et al._, _Endocr. Rev._, _5_:309-355 (1984)).  Other antigens of lesser, or uncertain, importance, include the second colloid antigen (Doniach, D.,
15   _et al._, _Clin. Endocrinol. Metab._ _8_:63-80 (1979)), tubulin (Rousset, B., _et al._, _Clin. Exp. Immunol._ _52_:325-332 (1983)), DNA (Katakura, M., _et al._, _J.Clin. Endocrinol. Metab._ _64_:405-408 (1987)) and Autoimmune Thyroid Disease-Related Antigen I (ATRA I) (Hirayu, H., _et al._, _J. Clin. Endocrinol. Metab._
20   _64_:578-584 (1987)).

Antibodies against the microsomal antigen, which is expressed on the cell surface (Khoury, E.L., _et al._, _Exp. Immunol._ _45_:315-328 (1981); Nilsson, M., _et al._, _Molec. Cell. Endocrinol._ _53_:177-185 (1987)), are believed to be of greater
25   importance than those against thyroglobulin in the pathogenesis of Hashimoto's thyroiditis.  This is because antimicrosomal antibodies (MSA) are more closely associated with the active phase of the disease (Volpe, R., In _Werner's The Thyroid_, 5th Edition (Ingbar, S.H., _et al._, Eds.), J.B.
30   Lippincott Co., Philadelphia, pp. 1266-1291 (1986); Bogner, U., _J. Clin. Endocrinol. Metab._ _59_:734-738 (1984); Jansson, R., _et al._, _Clin. Exp. Immunol._ _63_:80-86 (1986)) and are complement-fixing (Khoury, E.L., _et al._, _Exp. Immunol._ _45_:315-

328 (1981)).  These antibodies are, therefore, likely to
initiate thyroid cellular damage.

A major recent discovery regarding Hashimoto's
thyroiditis is that the previously ill-defined microsomal
antigen is, at least in part, thyroid peroxidase (TPO), the
primary enzyme involved in thyroid hormone synthesis.  This
conclusion was based on immunologic evidence (Czarnocka, B.,
FEBS Letters 109:147-152 (1985); Portmann, L., et al., J.
Clin. Endocrinol. Metab. 61:1001-1003 (1985); Mariotti, S.,
et al., J. Clin. Endocrinol. Metab. 65:987-993 (1987)) and
subsequently confirmed by the molecular cloning of the cDNA
for these proteins (Magnusson, R.P., et al., J. Biol. Chem.
262:13885-13888 (1987); Magnusson, R.P., et al., Mol.
Endocrinol. 1:856-861 (1987); Libert, R., et al., EMBO J.
6:4193-4176 (1987); Kimura, S., et al., Proc. Natl. Acad. Sci.
USA 84:5555-5559 (1987)) and the discovery that their derived
amino acid sequences are the same (Libert, R., et al., EMBO J.
6:4193-4176 (1987); Seto, P., et al., J. Clin. Invest.
80:1205-1208 (1987)).

Prior to the present invention, a suitable preparation of
recombinant TPO has not been available for studies on the
presumed abnormalities in immune regulation in Hashimoto's
thyroiditis, or for the demonstration of the specific B-cell
and T-cell epitopes involved in this disease.  In this
respect, understanding of the molecular mechanisms involved in
the pathogenesis of Hashimoto's thyroiditis lags far behind
that of other immune disorders, such as myasthenia gravis, a
disease for which pure antigen (the acetylcholine receptor)
has been obtained and epitopes already defined (Tzartos, S.J.,
et al., Proc. Natl. Acad. Sci. USA 85:2899-2903 (1988);
Hohlfeld, R., et al., J. Clin. Invest. 81:657-660 (1988)).

Human TPO (hTPO) immunopurified by monoclonal antibodies
(mAbs) has been available, but is of limited value because of:
(a) inadequate supplies of human thyroid tissue; (b) the

difficulties in purification of this membrane-bound antigen; and (c) contamination with other thyroid autoantigens such as thyroglobulin, which is highly abundant.

Fragments of hTPO have been generated as recombinant bacterial (β-galactosidase) fusion proteins, and reactivity of a number of Hashimoto patient sera with small fragments of TPO expressed as fusion proteins has been reported (Libert, R., et al., EMBO J. 6:4193-4176 (1987). Those data, however, are difficult to interpret, because the plaque assays used require extensive pre-adsorption of polyclonal antisera (Hirayu, H., et al., J. Clin. Endocrinol. Metab. 64:578-584 (1987)) and can yield false positive results.

For example, a reported fusion protein originally described as reactive with 19 of 20 Hashimoto patient sera (Libert, R., et al., EMBO J. 6:4193-4176 (1987), clone C2) has, upon immunopurification with anti-β-galactosidase mAbs, been found to react with fewer Hashimoto patient sera in an ELISA assay (Dinsart, C., et al., 17th Annual Meeting of the European Thyroid Association, Abstract #235 (1988)).

Thus, bacterial fusion proteins, too, have been of limited value because: (a) no combination of fragments has been found that reacts with all Hashimoto's sera; (b) the conformation of the fusion protein may differ from that of the native protein; and (c) the bacterial products may be toxic when added to immune cells in culture.

## SUMMARY OF THE INVENTION

In order to obtain full-length hTPO free of other potential thyroid antigens, the present inventor achieved expression of recombinant hTPO in non-thyroidal eukaryotic cells. Like native hTPO, this recombinant hTPO is enzymatically active, is expressed on the cell surface, and is not a fusion protein.

The recombinant hTPO of this invention is recognized in a specific manner by sera from patients with Hashimoto's thyroiditis that contain "antimicrosomal" antibodies. All 36 Hashimoto patient sera selected to represent a range of antimicrosomal antibody levels seen in this disease were reactive with the eukaryotic-expressed recombinant hTPO of the invention.

It is an object of the present invention, then, to provide for a convenient and economical source of recombinant hTPO, which does not suffer from the disadvantages associated with the immuno-purified native protein or with the recombinant fusion protein previously available. The present invention thus provides a number of important advances in the characterization of the human thyroid microsomal antigen, and opens the way to substantial further developments in this field.

Recombinant, enzymatically-active, human thyroid peroxidase has been generated in non-thyroidal eukaryotic cells. Unlike bacterial fusion proteins previously reported, the conformation of this protein is not encumbered by the β-galactosidase fusion partner. Furthermore, unlike the bacterially-produced protein, the TPO is glycosylated. The demonstration of functional TPO activity indicates unequivocally that the cDNA previously cloned (Magnusson, R.P., et al., J. Biol. Chem. 262:13885-13888 (1987); Magnusson, R.P., et al., Mol. Endocrinol. 1:856-861 (1987); Libert, R., et al., EMBO J. 6:4193-4176 (1987); Kimura, S., et al., Proc. Natl. Acad. Sci. USA 84:5555-5559 (1987)), is indeed TPO.

The present invention also provides for the identification of the β-cell epitope on thyroid peroxidase associated with autoimmune thyroid disease. In addition, this aspect of the invention provides a method for identifying the

molecular interaction responsible for the β-cell recognition
of thyroid peroxidase.

Experiments using the recombinant hTPO of the invention
expressed in a non-thyroidal eukaryotic cell prove that TPO,
independent of any other potential thyroid antigen, is a major
autoantigen in Hashimoto's thyroiditis. Thus, all 36
Hashimoto's patient sera tested reacted specifically with
recombinant hTPO in an approximately quantitative manner as
demonstrated by Western blot analysis. While previous
immunological studies strongly suggested that antimicrosomal
antibodies react with hTPO (Czarnocka, B., FEBS Letters
109:147-152 (1985); Portmann, L., et al., J. Clin. Endocrinol.
Metab. 61:1001-1003 (1985); Kotani, T., et al., J. Clin.
Endocrinol. Metab. 62:928-933 (1986); Mariotti, S., et al., J.
Clin. Endocrinol. Metab. 65:987-993 (1987)), it had been
difficult to exclude the possibility of contamination of the
immunopurified hTPO antigen by other, unidentified, thyroid
antigens. The only thyroidal (or, indeed, human) protein
produced by, or found in, the CHO-TPO cells of the present
invention is hTPO. Even though human sera from both normal
subjects and patients with Hashimoto's thyroiditis contain
antibodies that react with some antigen(s) of untransfected
CHO cells, only the Hashimoto's patient sera react with the
recombinant hTPO.

The present invention also sheds light on previous
observations that the microsomal antigen appeared as a doublet
when analyzed by polyacrylamide gel electrophoresis (PAGE) and
Western blot (Portmann, L., et al., J. Clin. Invest. 81:1217-
1224 (1988); Hamada, N., et al., J. Clin. Endocrinol. Metab.
61:120-128 (1985); Hamada, N., et al.,) J. Clin. Invest.
79:819-825 (1987). It was not known whether the doublet
represented two separate proteins or the partial degradative
product of a single protein. Kimura et al. observed two forms
of hTPO mRNA and cDNA, and suggested the possibility of

alternate splicing of the initial TPO transcripts (Kimura, S., et al., Proc. Natl. Acad. Sci. USA 84:5555-5559 (1987)). Nagayama et al. reported the existence of four different forms of hTPO mRNA transcripts in cultured Graves' thyroid cells after TSH stimulation (Nagayama, Y., et al., International Thyroid Symposium, Tokyo, Japan, Abstract #42 (1988)). The present discovery of a doublet as the product of a single, intron-less, hTPO gene argues strongly against the likelihood of alternate splicing.

The apparent conversion of the doublet to a single band after protein reduction, reminiscent of the data of Portmann et al., with a crude human thyroid extract (Portmann, L., et al., J. Clin. Invest. 81:1217-1224 (1988)), suggests that membrane-bound hTPO is linked through disulfide bonds to another, unidentified protein. An alternate interpretation, in line with the model of Taurog et al. (Yokoyama, N., et al., Mol. Endocrinol. 2:838-844 (1988)), is that intrachain disulfide bonds within TPO may alter the gel migratory behavior of TPO, resulting in the appearance of multiple forms. In contrast to observations of human thyroid microsomes in which the primary antigen (under non-reducing conditions) was 107 kD in size (Hamada, N., et al., J. Clin. Endocrinol. Metab. 61:120-128 (1985)), the present inventor observed, under the same conditions, that the major immunogenic form of recombinant hTPO in transfected CHO cells is about 200 kD in mass which is converted upon reduction to a single band of about 110 kD. This difference may be related to varied expression of hTPO in different cell types (human and CHO). However, it was also reported that a 200 kD protein was produced by subjecting the extracted human thyroid microsomal 107 kD protein major band to PAGE under non-reducing conditions (Hamada, N., et al., J. Clin. Endocrinol. Metab. 61:120-128 (1985)). Also, the present finding of a diminished 110 kD signal after reduction of the recombinant

hTPO protein is in accordance with other findings using the native microsomal antigen (Gardas, A., *et al.*, *J. Endocrinol. Invest.* *11*:385-388 (1988); Nakajima, Y., *et al.*, *Mol. Cell. Endocrinol.* *53*:15-23 (1987)).  Thus, in its native state, human TPO exists either as a multimer or in association with another membrane protein of similar size.  Epitope recognition by autoantibodies may be conformation-dependent.

The derived amino acid sequence of hTPO suggested to the present inventor the presence in recombinant full-length hTPO and thus, in naturally-occurring hTPO, of a signal peptide, as well as a putative hydrophobic membrane-spanning region (transmembrane domain) at the carboxyl terminus of the protein (amino acid residues 846-870) (Magnusson, R.P., *et al.*, *J. Biol. Chem.* *262*:13885-13888 (1987); Magnusson, R.P., *et al.*, *Mol. Endocrinol.* *1*:856-861 (1987); Kimura, S., *et al.*, *Proc. Natl. Acad. Sci. USA* *84*:5555-5559 (1987); Libert, F., *et al.*, *Nucl. Acids Res.* *15*:6735 (1987)).  Naturally-occurring hTPO has been shown to be a thyroidal cell surface protein.  Recombinant, enzymatically active hTPO is also cell membrane-associated in stably transfected non-thyroidal eukaryotic cells (Kaufman, K.D., *et al.*, *J. Clin. Invest.* *84*:394-403 (1989)).

While not intending to be bound by a particular theory, the present inventor hypothesized that the signal peptide directs the human TPO through the cell membrane, but that the hydrophobic region of hTPO becomes embedded in the cell membrane, thereby preventing secretion from the cell.

There has heretofore been no functional proof that the hTPO hydrophobic region 846-870 corresponds to a transmembrane domain.  The present invention demonstrates the existence of a transmembrane domain in hTPO, and that hTPO is predominantly an enzyme with an extracellular orientation.  The insertion, by site-directed mutagenesis, of a stop codon immediately upstream of this putative transmembrane domain converts hTPO

into a secreted protein that is enzymatically active and
immunologically intact.   By introducing the stop codon, the
hTPO was truncated by 85 residues, removing the carboxyl
terminus (933 amino acids).   Mutated   hTPO   cDNA,   inserted
5    into a eukaryotic expression vector, was stably transfected
into CHO cells.   Immunoprecipitation and PAGE of cellular $^{35}S$-
methionine-labeled proteins with Hashimoto's patient serum
revealed a 105-101 kD doublet.   In contrast, cells transfected
with wild-type hTPO yielded a 112-105 kD doublet.

10        In pulse-chase experiments, CHO cells expressing the
truncated hTPO protein secreted immunoprecipitable TPO into
the culture medium after 4 hours of chase, with levels
accumulating progressively over a 24 hour period.   In
contrast, CHO cells expressing wild-type hTPO released no im-
15   munoprecipitable TPO into the culture medium.   The secreted,
truncated form of hTPO appeared as a single band of lesser
electrophoretic mobility, as opposed to the doublet expressed
within cells.   TPO enzymatic activity was present in
conditioned medium from CHO cells transfected with the mutated
20   hTPO, but was absent in conditioned medium from cells
expressing wild-type hTPO.   The stability of the mutated
protein appeared similar to that of wild-type hTPO.

The secreted form of hTPO can be used to generate large
amounts of soluble TPO protein for use in structural and
25   immunological studies, as well as for diagnostic uses.

Thus, in one embodiment, there is provided according to
the invention recombinant, enzymatically active, TPO, or a
functional or chemical derivative thereof.

In another embodiment is provided hTPO produced by non-
30   thyroidal eukaryotic cells.

In another embodiment there is provided according to the
invention recombinant hTPO that is enzymatically active,
immunologically intact and secretable, or a functional or
chemical derivative thereof.

Yet another embodiment of the invention comprises a
plasmid selected from the group consisting of pECE-HTPO,
pHTPO(M1)-ECE-SV2-DHFR, pHTPO-DHFR-2B, pHTPO-DHFR-4C and
pHTPO-DHFR-4C-MTX.

5      There is also provided according to the invention a non-
thyroidal eukaryotic cell transformed with any of these
plasmids, as well as methods of producing hTPO comprising
culturing the transformed cell under conditions allowing
expression of the hTPO and recovering the hTPO.

10      In yet another embodiment, the invention provides for an
antibody against the hTPO of the invention.

Further, a method of detecting hTPO in a sample is
provided according to the present invention, comprising
contacting the sample with an antibody against full-length
15 recombinant hTPO or an antibody against a secretable hTPO,
wherein the antibody is detectably labeled, so as to form a
complex between the hTPO in the sample and the detectably
labeled antibody, and detecting the complexed or uncomplexed
labeled antibody.

20      In an additional embodiment, there is provided a kit for
the detection of hTPO in a sample, comprising container means
comprising one or more containers, wherein one of the
containers comprises detectably labeled antibody against hTPO.

Further, a method of detecting antibodies to hTPO in a
25 sample is provided according to the present invention,
comprising contacting the sample with full-length recombinant
hTPO or secretable recombinant hTPO so as to form a complex
between an hTPO-specific antibody in the sample and the
recombinant hTPO, and detecting the complexed antibody. In an
30 additional embodiment, there is provided a kit for the
detection of antibodies to hTPO in a sample, comprising
container means comprising one or more containers, wherein one
of said containers comprises recombinant hTPO.

These and other non-limiting embodiments of the present invention will be apparent to those of skill from the following detailed description of the invention.

5 **BRIEF DESCRIPTION OF THE DRAWINGS**

Figure 1. Construction of the expression plasmid pHTPO-ECE. pHTPO-BS (upper right) was digested with Not I, the ends blunted with the Klenow fragment of DNA polymerase I, and the DNA subsequently digested with Xba I. The 10 released Bluescript vector was further digested with Sca I to obtain good separation on agarose gel electrophoresis because of the similar size of this vector (2.95 kb) and the HTPO cDNA fragment (3.1 kb). The mammalian expression vector pECE (Ellis, L., et al., Cell 45:721-732 (1986)) 15 (upper left) was digested with Eco RI, the ends blunted with the Klenow fragment of DNA polymerase I, and the DNA subsequently digested with Xba I. The digested pHTPO-BS and pECE fragments were then ligated using T4 DNA ligase (Maniatis, T., et al., Molecular Biology: A Laboratory 20 Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1982)). The resulting plasmid, pHTPO-ECE (bottom), was transfected into competent XL1-Blue cells (Stratagene, San Diego, CA). Narrow hatching: HTPO; wide hatching: SV40; black: Multiple Cloning Sites.

25 Figure 2. Fluorescence-activated cell sorter (FACS) analysis of CHO cells transfected with pHTPO-ECE. CHO-HTPO12b cells were processed as described herein.

Panel A: Cells exposed to phycoerythrin (PE)-labeled second antibody alone, without prior exposure to human 30 serum.

Panel B: Cells incubated in serum (1:100) from a patient with Hashimoto's thyroiditis (ELISA value of 1.779) without subsequent incubation in PE-labeled second antibody.

Panel C: Cells sequentially incubated in the 35 Hashimoto's serum described in panel B and in PE-labeled second antibody.

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Panel D:  As in panel C, except that serum from a normal
individual, lacking antimicrosomal antibodies, was used.

Panels E and F:   The same data as in panels C and D
plotted to show the forward scatter. These data indicate that
5      the relative sizes of the cell populations reacting with the
normal and the Hashimoto's sera are the same.

Figure 3.   Linear regression analysis of ELISAs using
antibodies  against  human  thyroidal  microsomes  or  against
recombinant human TPO.

10     Figure 4. Linear regression analysis of ELISAs using
antibodies  against  human  thyroidal  microsomes  or  against
recombinant human TPO, 1/1000 dilution.  "Cardiff" refers to
the source of the microsomal antigen of both Figures 3 and 4.

Figure 5. Relative TPO activities observed in CHO cells
15     transfected with pECE-HTPO, pHTPO-DHFR-2B and pHTPO-DHFR-4C,
shown plotted against methotrexate concentration.

Figure 6.   Nucleotide sequence of human TPO gene after
site-directed mutagenesis.   The mutations incorporated two
stop codons, as well as an EcoRl site for confirmation, in the
20     region immediately upstream from the transmembrane region of
the human TPO gene.

Figure 7.   cDNA sequence and derived amino acid sequence
of human thyroid peroxidase (Magnusson, R.P., et al., Mol.
Endocrinol. 1:856-861 (1987)).

25     Figure 8.    Schematic  diagram  showing  the  expression
plasmid pHTPO(M1)-ECE-SV2-DHFR.

Figure 9.  Construction of the plasmid pHTPO(M1)-ECE-SV2-
DHFR.

Figure 10. Comparison of 51 sera, selected to provide a
30     spectrum of anti-MSA levels, in terms of their reactivity with
Graves'  thyroid  microsomes  and  recombinant,  enzymatically-
active human TPO generated in non-thyroidal eukaryotic cells.
The  anti-MSA  assay  data  are  expressed  as  an  ELISA  index,
relative to a standard serum.  Data for the anti-hTPO antibody

assay are expressed as absolute O.D. units, normalized to a blank well value of 0.000. (A) serum dilution 1/100 (sera from four normal patients are enclosed within the rectangle); (B) serum dilution 1/1,000; (C) serum dilution 1/10,000.

Figure 11. Two sera (#11 and 27) reacting discrepantly with human thyroid microsomes (A) and recombinant hTPO (B) are reacting with an antigen other than hTPO in panel A at standard (1/100) serum dilution. Dilution curves are also shown for two other sera (#12 and 28) with similar anti-MSA activity at standard serum dilution.

Figure 12. Intra-assay variability of anti-hTPO antibody ELISA at standard (1/100) serum dilution. Mean ± standard deviation of 10 iterations of anti-hTPO antibody ELISA results for three autoimmune sera selected to represent low, medium, and high autoantibody levels.

Figure 13. Confirmation, by nucleotide sequencing, of the mutations introduced into hTPO by site-directed mutagenesis. The nucleotide positions referred to correspond to those reported for human TPO (Rousset, B., et al., Clin. Exp. Immunol. 52:325-332 (1983)). TGA (2629-2631 bp) and TAG (2641-2643 bp) stop codons, as well as the EcoRI site, in the mutated hTPO-M1 are shown on the right. The nucleotide sequence of wild-type hTPO is shown on the left.

Figure 14. (A) Immunoprecipitation of mutated hTPO in different clones of transfected CHO cells. CHO - non-transfected CHO cells; CHO-TPO - CHO cells transfected with wild type hTPO; CHO-TPO-M1-POOLED - pooled colonies of CHO cells transfected with the mutated form of hTPO; CHO-TPO-M1-D through K - individual colonies of CHO cells, transfected with mutated hTPO, that were selected with cloning cylinders and then expanded. Cells were radiolabeled with ^{35}S-methionine and immunoprecipitated with Hashimoto's thyroiditis serum containing high anti-hTPO antibody levels.

(B) Immunoprecipitation of mutated hTPO from clones of CHO-TPO-M1-K cells generated by limiting dilution. Immunoprecipitations were performed with serum from a patient with Hashimoto's thyroiditis with high anti-hTPO antibody levels. The specificity of the immunoprecipitation is shown by the inability of serum from a normal individual (CON) to precipitate the 105-101 kD doublet.

Figure 15. Biosynthesis and processing of TPO. Immunoprecipitation studies were performed with CHO cells expressing wild-type hTPO (upper panel), and with CHO cells transfected with the mutated form of hTPO (lower panel). Pulse for 4 h (0 hours of chase) with $^{35}S$-methionine was followed by chase with unlabeled methionine for the indicated periods of time. Immunoprecipitations were then performed on both cell lysates and conditioned media, as indicated.

Figure 16. Human TPO enzymatic activity in the medium of CHO cells after transfection with wild-type hTPO (cell line CHO-TPO 12g) (Kotani, T., et al., J. Clin. Endocrinol. Metab. 62:928-933 (1986)) and CHO cells transfected with the mutated form of hTPO (CHO-TPO-M1-K1). Media were collected after 3 days of culture. TPO enzymatic activity in the media was measured by the guaiacol assay. The time course shown refers to the accumulation of oxidized guaiacol substrate in the assay, and not to the kinetics of enzyme secretion into the medium.

Figure 17. (A) T cell clones from the thyroid infiltrate in Graves' disease, expanded in the absence of antigen, recognize recombinant TPO. Clone + autologous irradiated PBL - black bars; clone + PBL + control (untransfected) CHO microsomes - striped bars; clone + PBL + CHO microsomes transfected with TPO - grey bars. Results are expressed as mean cpm of [$^3$H]thymidine incorporation from triplicate cultures. Error bars indicate standard errors of the mean

(S.E.M).    Similar  results  were  obtained  in  three  or  more
replicate experiments.

            (B)     Peripheral  blood  lymphocytes  from  both
5      patients and normal subjects proliferate in response to both
control  and  TPO-transfected  microsomes.    PBL  alone  -  black
bars;  PBL  +  control  microsomes  -  striped  bars;  PBL  +  TPO
transfected  microsomes  -  grey  bars.   Results  are  expressed  as
mean  cpm  $[^3H]$thymidine  incorporation  of  triplicate  cultures
10     (Error bars indicate S.E.M.)  81 - patient from whom T cell
clones  in  Fig.  17A  were  derived;  RG  -  another  female  with
Graves'  disease;  KH  -  normal  control  female.   Similar  results
have  been  obtained  from  other  individuals  in  separate
experiments.

15          Figure 18.   Determination of the epitope for the anti-
microsomal/TPO  monoclonal  antibody  20.10.   The  nucleotide
sequences of the 5'- and 3'-ends were determined for 14 clones
selected  from  the  hTPO  cDNA  fragment  library.    These
boundaries  are  annotated  by  the  numbers  assigned  to  the
nucleotides  in  hTPO  previously  reported  (Magnusson,  R.P.,  et
20     al., Mol. Endocrinol. 1:856-861 (1987)).  The smallest region
of  overlap  between  all  14  clones  is  from  881-927  b.p.   The
first  two  nucleotides  in  this  span  do  not  constitute  a
complete codon, so the epitope area can be defined as between
883-927 b.p., corresponding to the derived amino acid sequence
25     shown.

          Figure 19.   Determination of the epitope recognized by
TPO MAb 47.  The nucleotide sequences of the 5'- and 3'-prime
ends were determined for 18 clones in the TPO cDNA fragment
library (see Materials and Methods) recognized by MAb 47.  The
30     smallest region of overlap between all 18 clones is from 2219-
2247 basepairs in the human TPO cDNA sequence, coding for the
indicated amino acids.

          Figure 20.   Western  blot  analysis  of  human  TPO,  using
TPO MAb.  Recombinant human TPO expressed in Chinese hamster

ovary cells was used as antigen under denaturing and reducing
conditions (see Materials and Methods).  After polyacrylamide
gel  electrophoresis  and  transfer  to  the  membranes,  the
membranes were probed with the indicated antibodies.  TPO MAb
1, 2, 9, 15, 18, 24, 30, 40, 47, 53, 59, 60 and 64 are mouse
MAbs generated against native undenatured human TPO (Ruf, J.,
et al., Endocrinology 125:1211-8 (1989)).  The controls (Con)
are mouse MAbs raised against denatured human TPO (Portmann,
L., et al., J. Clin. Invest. 81:1217-1224 (1988)) (A and B)
and control mouse ascitic fluid (C).  The sizes of the mol wt
markers are shown on the left, and that of recombinant human
TPO is indicated by the arrow.

Figure descriptions continued at page 144.

### DESCRIPTION OF THE PREFERRED EMBODIMENTS

     In the following description, reference will be made to
various methodologies known to those of skill in the art of
molecular biology and immunology.  Publications and other
materials setting forth such known methodologies to which
reference is made are incorporated herein by reference in
their entireties as though set forth in full.

     Standard  reference  works  setting  forth  the  general
principles of recombinant DNA technology include Watson, J.D.
et al., Molecular Biology of the Gene, Volumes I and II, The
Benjamin/Cummings Publishing Company, Inc., publisher, Menlo
Park, CA (1987); Darnell, J.E. et al., Molecular Cell Biology,
Scientific American Books, Inc., publisher, New York, N.Y.
(1986); Lewin, B.M., Genes II, John Wiley & Sons, publishers,
New York, N.Y. (1985); Old, R.W., et al., Principles of Gene
Manipulation:   An  Introduction  to  Genetic  Engineering, 2d
edition, University of California Press, publisher, Berkeley,
CA (1981); and Maniatis, T., et al., Molecular Cloning:   A
Laboratory Manual, Cold Spring Harbor Laboratory, publisher,
Cold Spring Harbor, NY (1982).

By "cloning" is meant the use of *in vitro* recombination techniques to insert a particular gene or other DNA sequence into a vector molecule. In order to successfully clone a desired gene, it is necessary to employ methods for generating
5    DNA fragments, for joining the fragments to vector molecules, for introducing the composite DNA molecule into a host cell in which it can replicate, and for selecting the clone having the target gene from amongst the recipient host cells.

By "cDNA" is meant complementary or copy DNA produced
10    from an RNA template by the action of RNA-dependent DNA polymerase (reverse transcriptase). Thus a "cDNA clone" means a duplex DNA sequence complementary to an RNA molecule of interest, carried in a cloning vector.

By "cDNA library" is meant a collection of recombinant
15    DNA molecules containing cDNA inserts which together comprise the entire genome of an organism. Such a cDNA library may be prepared by methods known to those of skill, and described, for example, in Maniatis *et al.*, *Molecular Cloning: A Laboratory Manual*, *supra*. Generally, RNA is first isolated
20    from the cells of an organism from whose genome it is desired to clone a particular gene. Preferred for the purposes of the present invention are mammalian, and particularly human, cell lines. A presently preferred vector for this purpose is the λ-ZAP vector.

25    By "vector" is meant a DNA molecule, derived from a plasmid or bacteriophage, into which fragments of DNA may be inserted or cloned. A vector will contain one or more unique restriction sites, and may be capable of autonomous replication in a defined host or vehicle organism such that the
30    cloned sequence is reproducible. Thus, by "DNA expression vector" is meant any autonomous element capable of replicating in a host independently of the host's chromosome, after additional sequences of DNA have been incorporated into the

autonomous element's genome.  Such DNA expression vectors include bacterial plasmids and phages.

By "substantially pure" is meant any antigen of the present invention, or any gene encoding any such antigen, which is essentially free of other antigens or genes, respectively, or of other contaminants with which it might normally be found in nature, and as such exists in a form not found in nature.  By "functional derivative" is meant the "fragments," "variants," "analogs," or "chemical derivatives" of a molecule. A "fragment" of a molecule, such as any of the cDNA sequences of the present invention, is meant to refer to any nucleotide subset of the molecule.  A "variant" of such molecule is meant to refer to a naturally occurring molecule substantially similar to either the entire molecule, or a fragment thereof. An "analog" of a molecule is meant to refer to a non-natural molecule substantially similar to either the entire molecule or a fragment thereof.

A molecule is said to be "substantially similar" to another molecule if the sequence of amino acids in both molecules is substantially the same.  Substantially similar amino acid molecules will possess a similar biological activity. Thus, provided that two molecules possess a similar activity, they are considered variants as that term is used herein even if one of the molecules contains additional amino acid residues not found in the other, or if the sequence of amino acid residues is not identical.  As used herein, a molecule is said to be a "chemical derivative" of another molecule when it contains additional chemical moieties not normally a part of the molecule.  Such moieties may improve the molecule's solubility, absorption, biological half life, etc.  The moieties may alternatively decrease the toxicity of the molecule, eliminate or attenuate any undesirable side effect of the molecule, etc.  Moieties capable of mediating such effects are disclosed, for example, in <u>Remington's</u>

Pharmaceutical Sciences, 16th ed., Mack Publishing Co., Easton, Penn. (1980).

Similarly, a "functional derivative" of a gene of the human TPO antigen of the present invention is meant to include "fragments," "variants," or "analogues" of the gene, which may be "substantially similar" in nucleotide sequence, and which encode a molecule possessing similar activity.

A DNA sequence encoding the human thyroid peroxidase of the present invention, or its functional derivatives, may be recombined with vector DNA in accordance with conventional techniques, including blunt-ended or staggered-ended termini for ligation, restriction enzyme digestion to provide appropriate termini, filling in of cohesive ends as appropriate, alkaline phosphatase treatment to avoid undesirable joining, and ligation with appropriate ligases. Techniques for such manipulations are disclosed by Maniatis, T., et al., supra, and are well known in the art.

By "secretion" of recombinant hTPO for the purposes of the present invention, it is meant that the recombinant hTPO expressed by a host cell is directed through and dissociated from the host cell membrane.

A nucleic acid molecule, such as DNA, is said to be "capable of expressing" a polypeptide if it contains nucleotide sequences which contain transcriptional and translational regulatory information and such sequences are "operably linked" to nucleotide sequences which encode the polypeptide. An operable linkage is a linkage in which the regulatory DNA sequences and the DNA sequence sought to be expressed are connected in such a way as to permit gene expression. The precise nature of the regulatory regions needed for gene expression may vary from organism to organism, but shall in general include a promoter region which, in prokaryotes, contains both the promoter (which directs the initiation of RNA transcription) as well as the DNA sequences

which, when transcribed into RNA, will signal the initiation
of protein synthesis.   Such regions will normally include
those 5'-non-coding sequences involved with initiation of
transcription and translation, such as the TATA box, capping
5        sequence, CAAT sequence, and the like.

If desired, the non-coding region 3' to the gene sequence
coding for the protein may be obtained by the above-described
methods.  This region may be retained for its transcriptional
termination regulatory sequences, such as termination and
10       polyadenylation.   Thus, by retaining the 3'-region naturally
contiguous to the DNA sequence coding for the protein, the
transcriptional termination signals may be provided.   Where
the transcriptional termination signals are not satisfactorily
functional in the expression host cell, then a 3' region
15       functional in the host cell may be substituted.

Two DNA sequences (such as a promoter region sequence and
a human thyroid peroxidase encoding sequence) are said to be
operably linked if the nature of the linkage between the two
DNA sequences does not (1) result in the introduction of a
20       frame-shift mutation, (2) interfere with the ability of the
promoter region sequence to direct the transcription of the
thyroid peroxidase gene sequence, or (3) interfere with the
ability  of  the  thyroid  peroxidase  gene  sequence  to  be
transcribed by the promoter region sequence.   A promoter
25       region would be operably linked to a DNA sequence if the
promoter were capable of effecting transcription of that DNA
sequence.   Thus, to express the protein, transcriptional and
translational signals recognized by an appropriate host are
necessary.

30       The present invention encompasses the expression of the
human thyroid peroxidase protein (or a functional derivative
thereof) in either prokaryotic or eukaryotic cells, although
eukaryotic  (and,  particularly,  non-thyroidal  eukaryotic)
expression is preferred.

Preferred prokaryotic hosts include bacteria such as E.
coli, Bacillus, Streptomyces, Pseudomonas, Salmonella,
Serratia, etc. The most preferred prokaryotic host is E.
coli. Other enterobacteria such as Salmonella typhimurium or
5      Serratia marcescens, and various Pseudomonas species may also
be utilized. Under such conditions, the protein may not be
glycosylated. The procaryotic host must be compatible with
the replicon and control sequences in the expression plasmid.

To express the human thyroid peroxidase protein (or a
10     functional derivative thereof) in a prokaryotic cell (such as,
for example, E. coli, B. subtilis, Pseudomonas, Streptomyces,
etc.), it is necessary to operably link the human TPO encoding
sequence to a functional prokaryotic promoter. Such promoters
may be either constitutive or, more preferably, regulatable
15     (i.e., inducible or derepressible). Examples of constitutive
promoters include the int promoter of bacteriophage λ, the bla
promoter of the β-lactamase gene of pBR322, and the CAT
promoter of the chloramphenicol acetyl transferase gene of
pBR325, etc. Examples of inducible prokaryotic promoters
20     include the major right and left promoters of bacteriophage λ
(P_L and P_R), the trp, recA, lacZ, lacI, and gal promoters of
E. coli, the α-amylase (Ulmanen, I., et al., J. Bacteriol.
162:176-182 (1985)) and the σ-28-specific promoters of B.
subtilis (Gilman, M.Z., et al., Gene 32:11-20 (1984)), the
25     promoters of the bacteriophages of Bacillus (Gryczan, T.J.,
In: The Molecular Biology of the Bacilli, Academic Press,
Inc., NY (1982)), and Streptomyces promoters (Ward, J.M., et
al., Mol. Gen. Genet. 203:468-478 (1986)). Prokaryotic
promoters are reviewed by Glick, B.R., (J. Ind. Microbiol.
30     1:277-282 (1987)); Cenatiempo, Y. (Biochimie 68:505-516
(1986)); and Gottesman, S. (Ann. Rev. Genet. 18:415-442
(1984)).

Proper expression in a prokaryotic cell also requires the
presence of a ribosome binding site upstream of the gene-

encoding sequence. Such ribosome binding sites are disclosed,
for example, by Gold, L., et al. (Ann. Rev. Microbiol. 35:365-
404 (1981)).

   Most preferred hosts are eukaryotic hosts including
5    yeast, insects, fungi, and mammalian cells either in vivo, or
in tissue culture. Mammalian cells provide post-translational
modifications to protein molecules including correct folding
or glycosylation at correct sites. Mammalian cells which may
be useful as hosts include cells of fibroblast origin such as
10   VERO or CHO-K1, or cells of lymphoid origin, such as the
hybridoma SP2/0-AG14 or the myeloma P3x63Sg8, and their
derivatives. CHO cells are presently preferred mammalian host
cells. COS cells also are convenient eukaryotic hosts for .
human thyroid peroxidase expression, as well as for study of
15   the regulation of human thyroid peroxidase expression.

   For a mammalian cell host, many possible vector systems
are available for the expression of human TPO. A wide variety
of transcriptional and translational regulatory sequences may
be employed, depending upon the nature of the host. The
20   transcriptional and translational regulatory signals may be
derived from viral sources, such as adenovirus, bovine
papilloma virus, Simian virus, or the like, where the
regulatory signals are associated with a particular gene
which has a high level of expression. Alternatively, pro-
25   moters from mammalian expression products, such as actin,
collagen, myosin, etc., may be employed. Transcriptional
initiation regulatory signals may be selected which allow for
repression or activation, so that expression of the genes can
be modulated. Of interest are regulatory signals which are
30   temperature-sensitive so that by varying the temperature,
expression can be repressed or initiated, or are subject to
chemical regulation, e.g., metabolite.

   Yeast provides substantial advantages in that it can also
carry out post-translational peptide modifications including

glycosylation. A number of recombinant DNA strategies exist
which utilize strong promoter sequences and high copy number
of plasmids which can be utilized for production of the
desired proteins in yeast. Yeast recognizes leader sequences
5  on cloned mammalian gene products and secretes peptides
bearing leader sequences (i.e., pre-peptides).

Further, by use of, for example, the yeast ubiquitin
hydrolase system, in vivo synthesis of ubiquitin-human TPO
fusion proteins may be accomplished. The fusion proteins so
10  produced may be processed in vivo or purified and processed in
vitro, allowing synthesis of the human TPO protein with a
specified amino terminus sequence. Moreover, problems
associated with retention of initiation codon-derived
methionine residues in direct yeast (or bacterial) expression
15  may be avoided. Sabin et al., Bio/Technol. 7(7): 705-709
(1989); Miller et al., Bio/Technol. 7(7): 698-704 (1989).

Any of a series of yeast gene expression systems
incorporating promoter and termination elements from the
actively expressed genes coding for glycolytic enzymes
20  produced in large quantities when yeast are grown in mediums
rich in glucose can be utilized. Known glycolytic genes can
also provide very efficient transcriptional control signals.
For example, the promoter and terminator signals of the
phosphoglycerate kinase gene can be utilized.

25  Production of human TPO or functional derivatives thereof
in insects can be achieved, for example, by infecting the
insect host with a baculovirus engineered to express human TPO
by methods known to those of skill. Thus, in one embodiment,
sequences encoding human TPO may be operably linked to the
30  regulatory regions of the viral polyhedrin protein (Jasny,
Science 238: 1653 (1987)). Infected with the recombinant
baculovirus, cultured insect cells, or the live insects
themselves, can produce the human TPO protein in amounts as
great as 20 to 50% of total protein production. When live

insects are to be used, caterpillars are presently preferred
hosts for large scale human TPO production according to the
invention.

As discussed above, expression of the human thyroid
5    peroxidase protein in eukaryotic hosts requires the use of
eukaryotic regulatory regions.  Such regions will, in general,
include a promoter region sufficient to direct the initiation
of RNA synthesis.  Preferred eukaryotic promoters include the
promoter of the mouse metallothionein I gene (Hamer, D., _et_
10   _al._, _J. Mol. Appl. Gen._ _1_:273-288 (1982)); the TK promoter of
Herpes virus (McKnight, S., _Cell_ _31_:355-365 (1982)); the SV40
early promoter (Benoist, C., _et al._, _Nature (London)_ _290_:304-
310 (1981)); the yeast _gal4_ gene promoter (Johnston, S.A., _et_
_al._, _Proc. Natl. Acad. Sci. (USA)_ _79_:6971-6975 (1982); Silver,
15   P.A., _et al._, _Proc. Natl. Acad. Sci. (USA)_ _81_:5951-5955
(1984)).  Of these, presently the most preferred is the SV40
promoter.

As is widely known, translation of eukaryotic mRNA is
initiated at the codon which encodes the first methionine.
20   For this reason, it is preferable to ensure that the linkage
between a eukaryotic promoter and a DNA sequence which encodes
the human TPO protein (or a functional derivative thereof)
does not contain any intervening codons which are capable of
encoding a methionine (i.e., AUG).  The presence of such
25   codons results either in a formation of a fusion protein (if
the AUG codon is in the same reading frame as human TPO
encoding DNA sequence) or a frame-shift mutation (if the AUG
codon is not in the same reading frame as the human TPO
encoding sequence).

30   The human TPO encoding sequence and an operably linked
promoter may be introduced into a recipient prokaryotic or
eukaryotic cell either as a non-replicating DNA (or RNA)
molecule, which may either be a linear molecule or, more
preferably, a closed covalent circular molecule.  Since such

molecules are incapable of autonomous replication, the
expression of the human TPO protein may occur through the
transient expression of the introduced sequence. Alter-
natively, permanent expression may occur through the integra-
5      tion of the introduced sequence into the host chromosome.

In one embodiment, a vector is employed which is capable
of integrating the desired gene sequences into the host cell
chromosome. Cells which have stably integrated the introduced
DNA into their chromosomes can be selected by also introducing
10     one or more markers which allow for selection of host cells
which contain the expression vector. The marker may provide
for prototrophy to an auxotropic host, biocide resistance,
e.g., antibiotics, or heavy metals, such as copper or the
like. The selectable marker gene can either be directly
15     linked to the DNA gene sequences to be expressed, or
introduced into the same cell by co-transfection. Additional
elements may also be needed for optimal synthesis of single
chain binding protein mRNA. These elements may include splice
signals, as well as transcription promoters, enhancers, and
20     termination signals. cDNA expression vectors incorporating
such elements include those described by Okayama, H., _Mol.
Cel. Biol._ 3:280 (1983).

In a preferred embodiment, the introduced sequence will
be incorporated into a plasmid or viral vector capable of
25     autonomous replication in the recipient host. Any of a wide
variety of vectors may be employed for this purpose. Factors
of importance in selecting a particular plasmid or viral
vector include:  the ease with which recipient cells that
contain the vector may be recognized and selected from those
30     recipient cells which do not contain the vector; the number
of copies of the vector which are desired in a particular
host; and whether it is desirable to be able to "shuttle" the
vector between host cells of different species. Preferred
prokaryotic vectors include plasmids such as those capable of

replication in _E. coli_ (such as, for example, p8R322, ColE1,
pSC101, pACYC 184, πVX.   Such plasmids are, for example,
disclosed by Maniatis, T., _et al._ (In:  _Molecular Cloning, A_
_Laboratory Manual_, Cold Spring Harbor Press, Cold Spring
Harbor, NY (1982)).  _Bacillus_ plasmids include pC194, pC221,
pT127, etc.  Such plasmids are disclosed by Gryczan, T. (In:
_The Molecular Biology of the Bacilli_, Academic Press, NY
(1982), pp. 307-329).  Suitable _Streptomyces_ plasmids include
plJ101 (Kendall, K.J., _et al._, _J. Bacteriol._ _169_:4177-4183
(1987)), and streptomyces bacteriophages such as φC31 (Chater,
K.F., _et al._, In:   _Sixth International Symposium on_
_Actinomycetales Biology_, Akademiai Kaido, Budapest, Hungary .
(1986), pp. 45-54).  Pseudomonas plasmids are reviewed by
John, J.F., _et al._ (_Rev. Infect. Dis._ _8_:693-704 (1986)), and
Izaki, K. (_Jpn. J. Bacteriol._ _33_:729-742 (1978)).

     Preferred eukaryotic plasmids include 8PV, vaccinia,
SV40, 2-micron circle, etc., or their derivatives.  Such
plasmids are well known in the art (Botstein, D., _et al._,
_Miami Wntr. Symp._ _19_:265-274 (1982); Broach, J.R., In:  _The_
_Molecular Biology of the Yeast Saccharomyces:  Life Cycle and_
_Inheritance_, Cold Spring Harbor Laboratory, Cold Spring
Harbor, NY, p. 445-470 (1981); Broach, J.R., _Cell_ _28_:203-204
(1982); Bollon, D.P., _et al._, _J. Clin. Hematol. Oncol._ _10_:39-
48 (1980); Maniatis, T., In:  _Cell Biology:  A Comprehensive_
_Treatise, Vol. 3, Gene Expression_, Academic Press, NY, pp.
563-608 (1980)).

     Once the vector or DNA sequence containing the con-
struct(s) has been prepared for expression, the vector or DNA
construct(s) may be introduced into an appropriate host cell
by any of a variety of suitable means, including such
biochemical  means  as  transformation,  transfection,
conjugation,  protoplast  fusion,  calcium  phosphate-
precipitation,  and  application  with  polycations  such  as
diethylaminoethyl (DEAE) dextran, and such mechanical means as

electroporation, direct microinjection, and microprojectile
(biolistic) bombardment (Johnston _et al._, _Science_ 240(4858):
1538 (1988)), etc.

After the introduction of the vector, recipient cells are
grown in a selective medium, which selects for the growth of
vector-containing cells.    Expression of the cloned gene
sequence(s) results in the production of the human TPO
protein, or in the production of a fragment of this protein.
This can take place in the transformed cells as such, or
following the induction of these cells to differentiate.

The expressed protein may be isolated and purified in
accordance with conventional conditions, such as extraction,
precipitation,  chromatography,  affinity chromatography,
electrophoresis, or the like.  For example, the cells may be
collected by centrifugation, or with suitable buffers, lysed,
and the protein isolated by column chromatography, for
example, on DEAE-cellulose, phosphocellulose, polyribocyti-
dylic acid-agarose, hydroxyapatite or by electrophoresis or
immunoprecipitation.    Alternatively,  the human TPO or
functional derivative thereof may be isolated by the use of
anti-human TPO antibodies.  Such antibodies may be obtained by
well-known methods, some of which as mentioned hereinafter.

ANTIBODIES SPECIFIC FOR hTPO

The term "antibody" (Ab) or "monoclonal antibody" (mAb)
as used herein is meant to include intact molecules as well as
fragments thereof (such as, for example, Fab and $F(ab')_2$
fragments) which are capable of binding an antigen.  Fab and
$F(ab')_2$ fragments lack the Fc fragment of intact antibody,
clear more rapidly from the circulation, and may have less
non-specific tissue binding of an intact antibody (Wahl _et
al._, _J. Nucl. Med._ 24:316-325 (1983)).

Antibodies according to the present invention may be
prepared by any of a variety of methods.  For example, cells

expressing the human TPO protein, or a functional derivative
thereof, can be administered to an animal in order to induce
the production of sera containing polyclonal antibodies that
are capable of binding human TPO.

5          In a preferred method, antibodies according to the
present invention are mAbs. Such mAbs can be prepared using
hybridoma technology (Kohler et al., Nature 256:495 (1975);
Kohler et al., Eur. J. Immunol. 6:511 (1976); Kohler et al.,
Eur. J. Immunol. 6:292 (1976); Hammerling et al., In:
10     Monoclonal Antibodies and T-Cell Hybridomas, Elsevier, N.Y.,
pp. 563-681 (1981)). In general, such procedures involve
immunizing an animal with human TPO antigen. The splenocytes :
of such animals are extracted and fused with a suitable
myeloma cell line. Any suitable myeloma cell line may be
15     employed in accordance with the present invention. After
fusion, the resulting hybridoma cells are selectively
maintained in HAT medium, and then cloned by limiting dilution
as described by Wands, J.R., et al. (Gastroenterology 80:225-
232 (1981). The hybridoma cells obtained through such a
20     selection are then assayed to identify clones which secrete
antibodies capable of binding the human TPO antigen.

           Antibodies according to the present invention also may be
polyclonal, or, preferably, region specific polyclonal
antibodies. Region specific polyclonal antibodies and methods
25     of using them are described in co-pending U.S. application
Serial Number 06/731,470, filed 07 May 1985, the specification
of which is incorporated herein by reference as though set
forth in full.

           Antibodies against human TPO according to the present
30     invention are well suited for use in standard immunodiagnostic
assays known in the art, including such immunometric or
"sandwich" assays as the forward sandwich, reverse sandwich,
and simultaneous sandwich assays. The antibodies may be used
in any number of combinations as may be determined by those of

skill without undue experimentation to effect immunoassays of
acceptable specificity, sensitivity, and accuracy for the
human TPO antigen or equivalents thereof.

5      Standard reference works setting forth general principles
of immunology include Roitt, I., Essential Immunology, Sixth
Ed., Blackwell Scientific Publications, Publisher, Oxford
(1988); Kimball, J. W., Introduction to Immunology, Second
Ed., Macmillan Publishing Co., Publisher, New York (1986);
Roitt, I., et al., Immunology, Gower Medical Publishing Ltd.,
10     Publisher, London, (1985); Campbell, A., "Monoclonal Antibody
Technology," in, Burdon, R., et al., eds., Laboratory
Techniques in Biochemistry and Molecular Biology, Volume 13,
Elsevier, Publisher, Amsterdam (1984); Klein, J., Immunology:
The Science of Self-Nonself Discrimination, John Wiley & Sons,
15     Publisher, New York (1982); and Kennett, R., et al., eds.,
Monoclonal Antibodies. Hybridoma:   A New Dimension in
Biological Analyses, Plenum Press, Publisher, New York (1980).

By "detecting" it is intended to include determining the
presence or absence of a substance or quantifying the amount
20     of a substance.   The term thus refers to the use of the
materials, compositions, and methods of the present invention
for qualitative and quantitative determinations.

The isolation of other hybridomas secreting mAbs of the
same specificity as those described herein can be accomplished
25     by the technique of anti-idiotypic screening.  Potocnjak, et
al., Science 215:1637 (1982).    Briefly, an anti-idiotypic
(anti-Id) antibody is an antibody which recognizes unique
determinants generally associated with the antigen-binding
site of an antibody.   An Id antibody can be prepared by
30     immunizing an animal of the same species and genetic type
(e.g. mouse strain) as the source of the mAb with the mAb to
which an anti-Id is being prepared. The immunized animal will
recognize and respond to the idiotypic determinants of the

immunizing antibody by producing an antibody to these idiotypic determinants (the anti-Id antibody).

By using an anti-Id antibody which is specific for iditoypic determinants on a given mAb, it is then possible to identify other B cell or hybridoma clones sharing that idiotype. Idiotypic identity between the antibody product of two clones makes it highly probable that the antibody products of the two clones recognize the same antigenic epitopes.

The anti-Id antibody may also be used as an "immunogen" to induce an immune response in yet another animal, producing a so-called anti-anti-Id antibody. The anti-anti-Id may be epitopically identical to the original mAb which induced the anti-Id.

Thus, by using antibodies to the idiotypic determinants of a mAb, it is possible to identify other clones expressing antibodies of identical specificity.

Accordingly, mAbs generated against the hTPO antigen may be used to induce anti-Id antibodies in suitable animals, such as BALB/c mice. Spleen cells from such immunized mice are used to produce anti-Id hybridomas secreting anti-Id mAbs. Futher, the anti-Id mAbs can be coupled to a carrier such as keyhole limpet hemocyanin (KLH) and used to immunize additional BALB/c mice. Sera from these mice will contain anti-anti-Id antibodies that have the binding properties of the original mAb specific for the hPTO epitope. The anti-Id mAbs thus have their own idiotypic epitopes, or "idiotopes" structurally similar to the epitope being evaluated, such—as hTPO.

For replication, the hybridoma cells of this invention may be cultivated in vitro or in vivo. Production of high titers of mAbs in vivo production makes this the presently preferred method of production. Briefly, cells from the individual hybridomas are injected intraperitoneally into pristane-primed BALB/c mice to produce ascites fluid

containing high concentrations of the desired mAbs.  MAbs of
isotype IgM or IgG may be purified from such ascites fluids,
or from culture supernatants, using column chromatography
methods well known to those of skill in the art.

5      Antibodies according to the present invention are
particularly suited for use in immunoassays wherein they may
be utilized in liquid phase or bound to a solid phase carrier.
In addition, the antibodies in these immunoassays can be
detectably labeled in various ways.

10      There are many different labels and methods of labeling
known in the art.  Examples of the types of labels which can
be used in the present invention include, but are not limited
to, enzymes, radioisotopes, fluorescent compounds, chemilumi-
nescent compounds, bioluminescent compounds and metal
15      chelates.  Those of ordinary skill in the art will know of
other suitable labels for binding to antibodies, or will be
able to ascertain the same by the use of routine
experimentation.  Furthermore, the binding of these labels to
antibodies can be accomplished using standard techniques
20      commonly known to those of ordinary skill in the art.

One of the ways in which antibodies according to the
present invention can be detectably labeled is by linking the
antibody to an enzyme.  This enzyme, in turn, when later
exposed to its substrate, will react with the substrate in
25      such a manner as to produce a chemical moiety which can be
detected as, for example, by spectrophotometric or fluoro-
metric means.  Examples of enzymes which can be used to
detectably label antibodies include malate dehydrogenase,
staphylococcal nuclease, delta-V-steroid isomerase, yeast
30      alcohol dehydrogenase, alpha-glycerophosphate dehydrogenase,
triose phosphate isomerase, biotin-avidin peroxidase,
horseradish peroxidase, alkaline phosphatase, asparaginase,
glucose oxidase, beta-galactosidase, ribonuclease, urease,

catalase, glucose-VI-phosphate dehydrogenase, glucoamylase and
acetylcholine esterase.

The presence of detectably labeled antibodies also can be
detected by labeling the antibodies with a radioactive isotope
which then can be determined by such means as the use of a
gamma counter or a scintillation counter. Isotopes which are
particularly useful for the purpose of the present invention
are $^3H$, $^{125}I$, $^{32}P$, $^{35}S$, $^{14}C$, $^{51}Cr$, $^{36}Cl$, $^{57}Co$, $^{58}Co$, $^{59}Fe$ and
$^{75}Se$.

It is also possible to detect the binding of detectably
labeled antibodies by labeling the antibodies with a
fluorescent compound. When a fluorescently labeled antibody
is exposed to light of the proper wave length, its presence
then can be detected due to the fluorescence of the dye.
Among the most commonly used fluorescent labeling compounds
are fluoroscein, isothiocyanate, rhodamine, phycoerythrin,
phycocyanin, allophycocyanin, o-phthaldehyde and
fluorescamine.

The antibodies of the invention also can be detectably
labeled using fluorescent emitting metals such as $^{152}Eu$, or
others of the lanthanide series. These metals can be attached
to the antibody molecule using such metal chelating groups as
diethylenetriaminepentaacetic acid (DTPA) or ethylenediamine-
tetraacetic acid (EDTA).

Antibodies also can be detectably labeled by coupling
them to a chemiluminescent compound. The presence of the
chemiluminescent-tagged antibody is then determined by
detecting the presence of luminescence that arises during the
course of the chemical reaction. Examples of particularly
useful chemiluminescent labeling compounds are luminal,
isoluminol, theromatic acridinium ester, imidazole,
acridinium salts, oxalate ester , and dioxetane.

Likewise, a bioluminescent compound may be used to label
the antibodies according to the present invention. Biolumi-

nescence is a type of chemiluminescence found in biological
systems in which a catalytic protein increases the efficiency
of the chemiluminescent reaction. The presence of a biolumi-
nescent antibody is determined by detecting the presence of
5      luminescence. Important bioluminescent compounds for purposes
of labeling include luciferin, luciferase and aequorin.

The antibodies and substantially purified antigen of the
present invention are ideally suited for the preparation of a
kit.   Such a kit may comprise a carrier means being
10     compartmentalized to receive in close confinement therewith
one or more container means such as vials, tubes and the like,
each of said container means comprising the separate elements
of the assay to be used.

The types of assays which can be incorporated in kit form
15     are many, and include, for example, competitive and non-
competitive assays.   Typical examples of assays which can
utilize the antibodies of the invention are radioimmunoassays
(RIA), enzyme immunoassays (EIA), enzyme-linked immunosorbent
assays (ELISA), and immunometric, or sandwich, immunoassays.
20     By the term "immunometric assay" or "sandwich immuno-
assay," it is meant to include simultaneous sandwich, forward
sandwich and reverse sandwich immunoassays. These terms are
well understood by those skilled in the art. Those of skill
will also appreciate that antibodies according to the present
25     invention will be useful in other variations and forms of
assays which are presently known or which may be developed in
the future.   These are intended to be included within the
scope of the present invention.

Forward sandwich assays are described, for example, in
30     United States Patents 3,867,517; 4,012,294 and 4,376,110.
Reverse sandwich assays have. been described, for example, in
United States Patents 4,098,876 and 4,376,110.

In the preferred mode for preforming the assays it is
important that certain "blockers" be present in the incubation

medium (usually added with the labeled soluble antibody). The "blockers" are added to assure that non-specific proteins, protease, or human antibodies to mouse immunoglobulins present in the experimental sample do not cross-link or destroy the
5    antibodies on the solid phase support, or the radiolabeled indicator antibody, to yield false positive or false negative results.    The selection of "blockers" therefore adds substantially to the specificity of the assays described in the present invention.

10       It has been found that a number of nonrelevant (i.e. non-specific) antibodies of the same class or subclass (isotype) as those used in the assays (e.g. $IgG_1$, $IgG_{2a}$, IgM, etc.) can be used as "blockers."  The concentration of the "blockers" (normally 1-100 $\mu g/\mu l$) is important, in order to maintain the
15    proper sensitivity yet inhibit any unwanted interference by mutually occurring cross reactive proteins in human serum. In addition, the buffer system containing the "blockers" needs to be optimized.  Preferred buffers are those based on weak organic acids, such as imidazole, HEPPS, MOPS, TES, ADA, ACES,
20    HEPES, PIPES, TRIS, and the like, at physiological pH ranges. Somewhat less preferred buffers are inorganic buffers such as phosphate, borate or carbonate.   Finally, known protease inhibitors should be added (normally at 0.01-10 $\mu g/ml$) to the buffer which contains the "blockers."

25       There are many solid phase immunoadsorbents which have been employed and which can be used in the present invention. Well known immunoadsorbents include glass, polystyrene, polypropylene, dextran, nylon and other materials, in the form of tubes, beads, and microtiter plates formed from or coated
30    with such materials, and the like. The immobilized antibodies can be either covalently or physically bound to the solid phase immunoadsorbent, by techniques such as covalent bonding via an amide or ester linkage, or by adsorption.   Those skilled in the art will know many other suitable solid phase

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immunoadsorbents and methods for immobilizing antibodies thereon, or will be able to ascertain such, using no more than routine experimentation.

For _in vivo_, _in vitro_ or _in situ_ diagnosis, labels such as radionuclides may be bound to antibodies according to the present invention either directly or by using an intermediary functional group. An intermediary group which is often used to bind radioisotopes which exist as metallic cations to antibodies is diethylenetriaminepentaacetic acid (DTPA). Typical examples of metallic cations which are bound in this manner are: $^{99m}Tc$, $^{123}I$, $^{111}IN$, $^{131}I$, $^{97}Ru$, $^{67}Cu$, $^{67}Ga$ and $^{68}Ga$. The antibodies of the invention can also be labeled with non-radioactive isotopes for purposes of diagnosis. Elements which are particularly useful in this manner are $^{157}Gd$, $^{55}Mn$, $^{162}Dy$, $^{52}Cr$ and $^{56}Fe$.

The hTPO-encoding DNA sequence of the present invention, or a fragment thereof, may be used as a DNA probe to isolate or detect complementary DNA sequences according to well-known hybridization methods. The human antigen genes may then be cloned and expressed in a host to give the human antigen. This human antigen may then be used in diagnostic assays for the corresponding autoantibody.

The antigen of the invention may be isolated in substantially pure form employing antibodies according to the present invention. Thus, an embodiment of the present invention provides for substantially pure hTPO, characterized in that it is recognized by and binds to the anti-hTPO antibodies of the present invention. In another embodiment, the present invention provides a method of isolating or purifying hTPO by forming a complex with one or more antibodies directed against hTPO.

The substantially pure hTPO of the present invention may in turn be used to detect or measure antibody to hTPO in a sample, such as serum or urine. Thus, one embodiment of the

present invention comprises a method of detecting the presence
or amount of antibody to hTPO in a sample, comprising
contacting the sample containing the antibody to hTPO with
detectably labeled hTPO, and detecting the label.

5          It will be appreciated that immunoreactive fractions and
immunoreactive analogs of hTPO also may be used. By the term
"immunoreactive fraction" is intended any portion of the hTPO
antigen which demonstrates an equivalent recognition by, or
binding to, an antibody directed against hTPO. By the term

10     "immunoreactive analog" is intended a protein which differs
from hTPO by one or more amino acids, but which demonstrates
an equivalent recognition by, or binding to, an anti-hTPO
antibody.

15     T CELLS SPECIFIC FOR TPO

           Autoimmune diseases are thought to result at least in
part due to persistent activation of T cells by self antigens
(Janeway, C., Nature 341: 482 (1989)).  In the case of
autoimmune thyroiditis, as in Hashimoto's thyroidits, such a

20     self antigen can be any epitope of TPO which is recognized by
a receptor on a T cell capable of helping a B cell make an
anti-TPO antibody, or a T cell involved in the autoimmune
process by any other known mechanism (see below).

           One approach to the treatment of autoimmune thyroid

25     diseases as contemplated by the present inventor focuses on
disrupting the action of T lymphocytes involved in the disease
process.  T cells are readily available from the thyroid, for
example in Graves' disease in the form of infiltrates
extracted from thyroidectomy specimens.  By studying such

30     infiltrates, it is possible to examine the antigenic
specificities of T cells selected in vivo for their pathogenic
relevance.

           For example, the infiltrating T cells (as well as T cells
present in the circulation and in lymphoid organs such as

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lymph nodes and spleen) can act as T helper (Th) cells, responding to TPO epitopes, and helping B cells make specific anti-TPO antibodies. Alternatively, or additionally, such T cells can mediate a cell-mediated immune response and act on
5      thyroid epithelial cells either directly or via the local release of cytokines. This may lead to destruction of thyroid epithelial cells, when cytotoxic T cells specific for TPO are activated, or via an inflammatory response mediated by a different T cell class.
10     Disruption of the activation or action of such T cells would serve to inhibit the production of anti-TPO antibodies, on the one hand, or of thyroid epithelium-damaging T cells on the other.

       One embodiment therefore provides peptides capable of
15     binding to the T cell receptor (TCR) of a TPO-specific T cell. Such TPO-related peptides include at least a portion of a T cell epitope of TPO (such as the NP-7 epitope of Example XII). Useful peptides include a sequence of about 5 or more amino acids of TPO, or derivatives of such peptides, which are
20     capable of binding to the TCR of a TPO-specific T cell. Acting as a competitive antagonist for the native autoantigen, such a peptide can inhibit antigen presentation to T cells, or other antigen-specific cell-cell (e.g., T-T or T-B) interactions in the immune system which are needed for
25     generation of either anti-TPO antibodies or TPO-specific cell-mediated immunity. (For discussion of such peptide-based approaches to immunotherapy of autoimmune disease, see, for example: Acha-Orbea, H., et al. (Ann. Rev. Immunol. 7:371-405 (1989); Kumar, V., et al., Ann. Rev. Immunol. 7:657-682
30     (1989); Urban, J.L. et al., Cell 54:577-592 (1989); Wraith, D.C., et al. (Cell 57:709-715 (1989); Wraith, D.C., et al., Cell 59:247-255 (1989); Urban, J.L., et al., Cell 59:257-271 (1989); and Janeway, C.A., Nature 341:482-483 (1989), all of which references are hereby incorporated by reference).

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Another embodiment of the invention provides for a pharmaceutical preparation comprising the above peptides. In yet another embodiment of the invention, a method of treating autoimmune disease, including but not limited to Hashimoto's thyroiditis, is provided which comprises administering to a patient suffering from such disease a pharmaceutical preparation comprising a TPO-related peptide.

An alternate peptide-based therapeutic strategy contemplated within the scope of the present invention is directed to vaccines comprising TPO-specific T cells (Cohen, I.R., Immunol. Rev. 94:5-21 (1986); Prog. Immunol. VI:491-499 (1986); Scientific Amer. 258:52-60 (1988); Hosp. Prac. pp. 57-64 (February 15, 1989); Cohen, I.R., et al., Immunol. Today 9:332-335 (1988)) and peptides mimicking the TCR of such TPO-specific T cells (Vandenbark, A.A. et al., Nature 341:541-544 (1989); Howell, M.D. et al., Science 246:668-671 (1989)). Such preparations are administered to an individual to prevent or suppress an autoimmune response to TPO by inducing a state of "counter-autoimmunity." Such counter-autoimmunity is thought to be mediated by T cells which are specific to the TCR of the autoimmune (i.e., TPO-specific) T cell (Cohen, supra, Vandenbark et al., supra, and Sun, D. et al., Nature 332:843-845 (1988); Europ. J. Immunol. 18:1993-1999 (1988)).

The invention is therefore directed to T cells specific for TPO capable of acting as a "vaccine" and inducing a state of counter-autoimmunity. Another embodiment includes TCR-mimicking peptides of such T cells. Yet another embodiment is directed to the T cells induced by such TPO-specific T cell and TCR peptide vaccines which mediate the counter-autoimmune effects or down-regulate TPO-specific T cells. Another embodiment of the invention provides for a pharmaceutical preparation comprising such a T cell vaccine, TCR peptide, or counter-autoimmune T cell. In yet another embodiment of the

current invention, a method of treating autoimmune disease,
such as Hashimoto's thyroiditis, is provided which includes
the use of a pharmaceutical preparation comprising either a
TPO-specific T cell vaccine, a TCR peptide vaccine, or a
5    counter-autoimmune T cell specific for TPO-specific T cells.

     An additional embodiment of the present invention is
directed to a T suppressor (Ts) lymphocyte capable of
interacting specifically with an anti-TPO B cell or T cell,
leading to suppression of an anti-TPO immune response. Such
10   suppression could be of TPO-specific antibody production or of
TPO-specific T cell-mediated thyroid damage such as that
mediated by cytotoxic T cells or in a TPO-specific delayed
hypersensitivity response.  Thus in one embodiment, the
invention is directed to an epitope of TPO capable of inducing
15   antigen-specific Ts cells and its use in generating Ts cells
and in treating autoimmune thyroiditis. Another embodiment is
a TPO-specific Ts in a pharmaceutical preparation.  Yet
another embodiment is directed to a method of treating
autoimmune thyroiditis, such as Hashimoto's disease,
20   comprising administering a pharmaceutical preparation
comprising a TPO epitope capable of inducing Ts cells.  An
additional embodiment is a method of treating autoimmune
thyroiditis by administering a pharmaceutical preparation
comprising TPO-specific Ts cells capable of suppressing an
25   anti-TPO response. For a discussion of suppressor cells, see,
for example, Green, D., *et al.*, *Ann. Rev. Immunol.* 1: 439
(1983) and Benacerraf, B., In: *The Biology of Immunologic*
*Disease*, HP Publishing Co., Inc., NY, pp. 49-62 (1983).

     The present invention allows the determination of the T
30   cell epitope or epitopes of TPO (see Example XII, below) using
standard techniques commonly known to those of ordinary skill
in the art. Further, the present invention makes possible the
characterization of the autoimmune TCR specific to the TPO
using methods described in, for example, Burns, F., *et al.*, *J.*

Exp. Med. 169: 27 (1989).  If the autoimmune T cells can be
eliminated or prevented from reacting with the TPO, the
effects of thyroiditis may be greatly alleviated.  T cells
that will accomplish this objective may be generated which are
5        specific for the autoimmune TCR for TPO using methods
described in, for example, Acha-Orbea, H., et al., Ann. Rev.
Immunol. 7: 371 (1989).

The manner and method of carrying out the present
10       invention may be more fully understood by those of skill by
reference to the following examples, which examples are not
intended in any manner to limit the scope of the present
invention or of the claims directed thereto.

15                                    EXAMPLE I
Construction of a Human Graves' Thyroid cDNA Library

A thyroid cDNA library was constructed to maximize the
inclusion of full-length cDNA in the coding orientation.
20       Hyperplastic thyroid tissue was obtained from a patient
undergoing thyroidectomy for Graves' disease.  mRNA was
isolated according to the method of Han et al. (Han, J.H., et
al., Biochem. 26:1617-1625 (1987)).  Double-stranded cDNA was
synthesized from 15 μg mRNA as described by Gubler and Hoffman
25       (Gubler, U., et al., Gene 2:263-269 (1983)).  Not I and Xba I
linker-primers/adaptors were incorporated into the cDNA to
create those restriction sites at the 5' and 3' ends,
respectively, of the cDNA (Han, J.H., et al., Biochem.
26:1617-1625 (1987)).  The cDNA was size-selected ( > 1 kb) by
30       agarose gel (Seaplaque, FMC, Rockland, ME) electrophoresis,
digested with Not I and Xba I, ligated into Not I- and Xba I-
cut bacteriophage lambda-Zap using T4 DNA ligase, and packaged
(Giga-Pak Gold, Stratagene, San Diego, CA).  The resulting

phage library contained a total of 2 x 10⁴ recombinant clones
before amplification.

### EXAMPLE II

5          ### Screening for Full-length Human TPO cDNA


The amplified cDNA library was plated at a density of 4
x 10⁴ pfu per 150 mm diameter dish and probed using the insert
from a partial human TPO cDNA clone (clone 19). Two positive
10    bacteriophage clones were isolated.  A Bluescript phagemid
containing the human TPO cDNA insert was generated from one of
these clones using the helper phage R408, according to the
Stratagene protocol.  The resulting recombinant Bluescript
plasmid (pHTPO-BS) contained bases 5-3060 of human thyroid
15    peroxidase cDNA, including the start of translation and the
poly-A tail.  DNA sequence was determined from this double-
stranded plasmid using the Sequenase kit and protocol (United
States Biochemical, Cleveland, OH).  Sequence within the cDNA
was confirmed to be identical to human TPO cDNA at the 5' and
20    3' ends and in the regions adjacent to 10 oligonucleotide
primers distributed throughout the cDNA (Magnusson, R.P., _et
al._, _Mol. Endocrinol._ _1_:856-861 (1987)).

### EXAMPLE III

25          ### Construction of pHTPO-ECE


The mammalian cell expression vector pECE (Ellis, L., _et
al._, _Cell_ _45_:721-732 (1986)) was obtained from Dr. William
Rutter (U.C.S.F.).   Human TPO cDNA was cloned into the
30    multiple cloning site of this vector as described in Figure 1.
Enzyme reactions and DNA manipulations were performed as
described in Maniatis et al. (Maniatis, T., _et al._, _Molecular
Biology:  A Laboratory Manual_, Cold Spring Harbor Laboratory,
Cold Spring Harbor, NY (1982)).

## EXAMPLE IV

### Transfection of Chinese Hamster Ovary Cells with pHTPO-ECE

5    Chinese hamster ovary cell line CHO-K1 was maintained in
Hams' F-12 medium supplemented with 10% fetal bovine serum,
penicillin (125 units/ml), streptomycin (100 µg/ml) and
amphotericin-B (2.5 µg/ml). Transfection and selection with
G-418 (GIBCO, Grand Island, NY) was carried out by the method
10   of Chen and Okayama (Chen, C., et al., Mol. Cell. Biol.
7:2745-2752 (1987)). 20 µg pHTPO-ECE plus 2 µg pSV2-neo (28)
(from Dr. John Baxter, U.C.S.F.) were used for the
transfection. Control transfections with 20 µg pECE plus 2 µg
pSV2-neo, and 20 µg pSV2-neo alone, were performed
15   concurrently.

## EXAMPLE V

### RNA Extraction and Northern Blot Analysis

20   Total cellular RNA was extracted by the method of
Chomczynski and Sacchi (Chomczynski, P., et al., Anal.
Biochem. 162:156-159 (1987)). 15 µg of RNA was
electrophoresed in formaldehyde gels as described by Maniatis
et al. (Maniatis, T., et al., Molecular Biology: A Laboratory
25   Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
(1982)). RNA was blotted onto a Zeta-Probe membrane (BioRad,
Richmond, CA) and probed with a 0.56 kb human TPO cDNA probe
(clone 31 insert), labeled to a specific activity of 4 X $10^9$
cpm/µg DNA using the Multi-Prime labelling kit from Amersham
30   (Arlington Heights, IL).

## EXAMPLE VI

### Western Blot Analysis

Transfected CHO cells were extracted to obtain soluble
protein. Five 100 mm diameter dishes were washed 3 times with
calcium-magnesium free phosphate-buffered saline (PBS). After
aspiration, 5 ml of 0.5% Triton X-100 in the same buffer,
supplemented with 10 µg/ml leupeptin, 0.5 mg/ml bacitracin and
2 mM phenylmethylsulfonyl fluoride (all from Sigma, St. Louis,
MO), were added to the first dish. This initial cell solution
was scraped and transferred successively to the other 4
dishes of cells. The cell solution was then tumbled for 1
hour at 4°C. After centrifugation for 3 minutes at 10,000 x
g, the supernatant was saved and stored at -20°C until use.
Protein content was determined (Bradford, M.M., Anal. Biochem.
72:248-254 (1976)) and 50 µg protein/lane electrophoresed on a
7.5% polyacrylamide SDS gel (Laemmli, U.K., Nature 227:680-689
(1970)). Proteins were electrotransferred (30 V x 5 hours, or
250 mA overnight) to nitrocellulose membranes (Schleicher and
Schuell, Keene, NH) in an electroblotting apparatus (Hoeffer,
San Francisco, CA) containing 25 mM Tris, 192 mM glycine, 20%
methanol. In later experiments, transfer was accomplished
using a Polyblot semi-dry electrotransfer system (American
Bionetic, Hayward, CA), according to the directions of the
manufacturer. Membranes were rinsed once in TBS (0.1 M Tris,
pH 8.0, 0.15 M NaCl), then for 30-60 minutes at room
temperature in TBS containing 0.5 % Tween 20 (Sigma, St.
Louis, MO). After 3 further rinses with TBS-Tween, the blots
were probed as described by Young and Davis (Young, R.A., et
al., In Genetic Engineering: Principals and Methods, Plenum
Publishing Corp., 7:29-41 (1985)) using a 1:250 dilution of a
mouse mAb against the thyroid microsomal antigen (Portmann,
L., et al., J. Clin. Invest. 81:1217-1224 (1988)), followed

by a 1:250 dilution of horseradish peroxidase-conjugated goat
anti-mouse IgG antibody (Sigma, St. Louis, MO).

In other experiments, CHO-HTP012b cell extracts were
probed using a panel of polyclonal Hashimoto's thyroiditis

5    sera, provided by Dr. S. M. McLachlan, University of Wales,
Cardiff.  Antimicrosomal antibody titers had previously been
determined by enzyme-linked immunosorbant assay (ELISA) in the
presence of excess thyroglobulin (Jansson, R., et al., Clin.
Exp. Immunol. 63:80-86 (1986)).  Multiple Hashimoto's

10   thyroiditis sera were applied to a single filter overnight at
4°C using a Miniblotter 45 manifold (Immunetics, Cambridge,
Mass.).  Membranes were then processed as described above,
except that alkaline phosphatase-conjugated goat anti-human
IgG, Fc fragment specific (Cappel, Organon Teknika Corp., West

15   Chester, PA) was used as the second antibody with nitroblue
tetrazolium (0.3 mg/ml) and 5-bromo-4-chloro-3-indolyl-
phosphate (0.15 mg/ml) in 100mM Tris, pH 9.5, 100 mM NaCl, 5
mM $MgCl_2$.

20                        EXAMPLE VII
       Fluorescence-activated Cell Sorter (FACS) Analysis

CHO-HTP012b cells were processed as described by Ellis et
al. (Ellis, L., et al., Cell 45:721-732 (1986)).  In brief,

25   cells from a 100 mm diameter dish were detached by mild
trypsinization, and the cells rinsed and pelleted (5 minutes
at 100 x g, 4°C) in Ham's F12 medium, 10% fetal calf serum
(see above).  The cells were resuspended in 0.2 ml of
phosphate-buffered saline (PBS), 10mM Hepes, pH 7.4, 0.05% Na

30   azide (buffer A).  Serum to be tested (2ul) was added for 30
minutes at 4°C, followed by two rinses in buffer A with 2%
fetal calf serum and resuspension in 0.2 ml of the same
solution. 25 ul of goat anti-human IgG, Fc specific, affinity-
purified, R-Phycoerythrin-labeled (Caltag, South San

Francisco, CA) were added for another 30 minutes at 4°C.
After 3 washes in buffer A, the cells were analyzed on a
fluorescence-activated cell sorter.

5                          **EXAMPLE VIII**
                **Assay of Human TPO Enzymatic Activity**

Human TPO activity was assayed following extraction from cell
microsomes with trypsin and deoxycholate as previously
10    described (Magnusson, R.P., et al., Endocrinol. 116:1493-1500
(1985)). In later experiments, a more rapid method was used.
Cells were suspended with a rubber scraper in 1.5 ml calcium-
magnesium free Dulbecco's phosphate-buffered saline and
protein determined on a 5 ul aliquot. The cells were then
15    pelleted in a microcentrifuge for 2 minutes. Cold 0.1%
deoxycholate (0.2 ml/mg cellular protein) was added for 10
minutes. The extract was microcentrifuged for 5 minutes and
the supernatant removed for assay. One guaiacol unit is
defined as a _A470 of 1.0 per minute which is equivalent to
20    150 nmols guaiacol oxidized per minute (Chance, B., et al.,
In Methods in Enzymology (Colowick, S.P., et al.), Academic
Press, New York 2:764-775 (1955)). One unit of iodide
peroxidase is defined as a _A353 of 1.0 per minute which
corresponds to 43 nmols I3- formed per minute (Magnusson,
25    R.P., et al., J. Biol. Chem. 259:13783-13790 (1984)).

                          **EXAMPLE IX**
       **Primary Culture of Human Graves' Disease Thyroid Cells**

30         Human Graves' disease thyroid tissue was dispersed and
the cells cultured as previously described (Hinds, W.E., et
al., J. Clin. Endocrinol. Metab. 52:1204-1210 (1981)). After
3 days in culture, fresh medium containing 12.5 mU/ml TSH was

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added for an additional 3 days before the cells were harvested
and extracted as described above for the western blots.

**EXAMPLE X**

5      Comparison of Recombinant hTPO and Microsomal Antigen as
   Sources of Antigen for ELISAs for Anti-MSA/Anti-TPO Antibodies

        Sera from 51 individuals were provided by Dr. S. M.
   McLachlan (University of Wales College of Medicine, Cardiff,
10   U.K.).   Forty seven of these sera were from patients with
   autoimmune thyroid disease, selected to represent a balanced
   spectrum of anti-MSA titers from low to very high.  Four sera
   were  from  normal  individuals.    Anti-MSA  and  anti-TGA
   antibodies were measured  by the method of Schardt et al.
15   (Schardt, C.W., et al., J. Immunol. Methods 55:155-168 (1982))
   and the method of Endo et al. (Endo, Y., et al., Clin. Chim.
   Acta 103:67-77 (1980)), as modified by McLachlan et al.
   (McLachlan, S.M., et al., Immunol. Letters 4:27-33 (1982)),
   respectively.    For  the  anti-MSA  assay,  human  thyroid
20   microsomes were prepared from frozen Graves' thyroid tissue
   obtained  at operation  for  the  treatment  of  this disease
   (Schardt,  C.W.,  et  al.,  J.  Immunol.  Methods  55:155-168
   (1982)).  In order to avoid cross-reactivity of patients' sera
   with . any  thyroglobulin  remaining  in  the  microsomal
25   preparation, sera were pre-adsorbed in buffer containing 100
   μg/ml (1.5 x 10$^{-6}$ M) thyroglobulin (obtained from the same
   tissue) at 4°C overnight and thereafter at room temperature
   for 2 hours before assay (Schardt, C.W., et al., J. Immunol.
   Methods 55:155-168 (1982)).
30        The  generation  of  Chinese  hamster  ovary  (CHO)  cells
   (clone CHO-HTPO 12b) expressing enzymatically-active human TPO
   has been described above.  These cells had been transfected
   with the recombinant plasmid pHTPO-ECE, constructed by the
   insertion of a full-length human TPO cDNA into the expression
35   vector  pECE.    CHO-HTPO  12b  and  CHO-K1  (control,  non-

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transfected) cells were grown in Ham's F-12 medium
supplemented with 100 g/L fetal bovine serum (FBS), penicillin
(125 units/ml), gentamicin (48 μg/ml) and amphotericin-B (2.5
μg/ml). Cells were grown to confluence in 100 mm dishes, the
5    cells were rinsed three times with Dulbecco's calcium-
magnesium free, phosphate-buffered saline (PBS), and then
scraped into a solution containing 10 mM Tris, pH 7.4, 0.25 M
sucrose, 2 mM phenylmethyl sulfonyl fluoride, 10 μg/ml
leupeptin, 0.5 mg/ml bacitracin (Buffer A). Cells were
10   homogenized for 20 seconds with a Polytron, centrifuged for 15
minutes at 10,000 x g, 4°C, and the supernatant then
centrifuged for 1 hour at 100,000 x g, 4°C. The microsomal
pellet was resuspended in 0.5 ml of Buffer A, homogenized in a
Dounce homogenizer, and then frozen at -80°C until use.
15   Protein content was determined by the method of Bradford
(Bradford, M.M., Anal. Biochem. 72:248-254 (1976)). Yield of
microsomal protein was approximately 100-200 μg per 100 mm
dish of confluent cells.

    Sera to be tested were stored in aliquots at -80°C before
20   use. The assay procedure was that of Schardt et al. (Schardt,
C.W., et al., J. Immunol. Methods 55:155-168 (1982)), with
slight modifications. Multiwell micro-ELISA plates (Dynatech
Labs, Chantilly, VA) were coated (overnight at 4°C) with 4 μg
CHO-HTPO 12b or CHO-K1 microsomal protein per well in coating
25   buffer (0.05 M sodium bicarbonate, pH 9.3, 0.02% sodium
azide). The wells were then rinsed twice in 0.2 M Tris, pH
7.4, 0.15 M NaCl (Tris buffer), once in 0.2 M Tris, pH 7.4,
0.15 M NaCl, 0.05% Tween 20 (Tris-Tween buffer), and once in
Tris buffer. 100 ul of PBS, 50 g/L bovine serum albumin (BSA)
30   (Sigma, St. Louis, MO) were added to each well and incubated
for 20 minutes at room temperature. After aspiration, the
wells were washed twice in Tris buffer, once in Tris-Tween
buffer, and once in Tris buffer.

Serum samples were diluted 1/100, 1/1000 or 1/10,000 in
PBS, 5 g/L BSA. 100 ul of the diluted serum sample were added
per well in duplicate and incubated for 1 hour at 37°C. The
wells were then washed three times with PBS.  100 ul of
5      peroxidase-conjugated, affinity-purified, goat anti-human IgG,
Fc fragment specific antibody (Cappel, Organon Teknika Corp.,
West Chester, PA.), diluted 1/500 in PBS, 250 g/L FBS, were
added to each well and incubated for 1 hour at 37°C.  The
wells were then washed four times with Tris-Tween buffer.
10     100 ul of substrate solution (12 ml of 0.23 M citrate, 0.26 M
sodium phosphate, pH 5.0 solution + 12 uL 30% $H_2O_2$ + 4.2 mg
ortho-phenylenediamine) were added to each well and incubated
for 30 minutes at room temperature. The reaction was stopped .
by adding 100 ul of 20% sulfuric acid to each well.  ELISA
15     values (OD 490 nm) were measured in a micro-ELISA reader and
normalized (blanked) to a well lacking antigen.


### EXAMPLE XI

#### Oligonucleotide-Directed Mutagenesis of Human TPO cDNA

20     A.  METHODS
The non-coding strand of human TPO cDNA, in the phagemid
Bluescript (Stratagene, San Diego, CA), was used as a template
for oligonucleotide-directed mutagenesis.  A 52 bp mutagenic
primer
25     (5'-AGGCTCCCTCGGGTGACTTGAATTCCCATGTAGCTGGCTGCTCTGCTGATCG-3'),
synthesized by the Molecular Genetics Core Facility, San
Francisco Veterans' Administration Medical Center, was
designed to generate two stop codons directly upstream of the
putative membrane-spanning region of the protein.  Thus, TGA
30     and TAG codons were created at 2629-2631 bp and 2641-2643 bp
in human TPO cDNA (Magnusson, R.P., et al., Mol. Endocrinol.
1:856-861 (1987)), respectively.  For convenient screening of
mutants, an Eco RI restriction site (GAATTC, at 2630-2635 bp)
was created together with the first (TGA) stop codon.  The

mutagenesis procedure was performed according to the protocol
of the manufacturer (Muta-gene phagemid in vitro mutagenesis
kit, Biorad, Richmond, CA) to generate the plasmid pHTPO(M1)-
BS.

5       After confirmation of the mutation by nucleotide
sequencing (Sanger, F., et al., Proc. Natl. Acad. Sci. USA
74:5463-5467 (1977)) (Fig. 13), the cDNA was excised by
digestion of pHTPO(M1)-BS with Not I, the ends blunted with
the Klenow fragment of DNA Polymerase I, and the cDNA
10      liberated by digestion with Xba I. The mutated cDNA (3.05 Kb)
was substituted for wild-type human TPO cDNA in the plasmid
pSV2-DHFR-ECE-HTPO, to generate pHTPO(M1)-ECE-SV2-DHFR. This
plasmid contains components of the expression vectors pECE
(Ellis, L., et al., Cell 45:721-732 (1986)) and pSV2-dhfr
15      (Lee, F., et al., Nature 294:228-232 (1981)), provided by Dr.
William Rutter (University of California, San Francisco) and
Dr. Gordon Ringold (Syntex, Palo Alto), respectively. In
brief, pSV2-DHFR-ECE-HTPO was digested with Sal I, the ends
blunted with the Klenow fragment of DNA polymerase I, and the
20      hTPO cDNA released by digestion with Xba I. The remaining
vector (pSV2-DHFR-ECE) was treated with bacterial alkaline
phosphatase, gel purified, and recovered in SeaPlaque agarose
(FMC BioProducts, Rockland ME). Mutated hTPO cDNA, also
recovered in SeaPlaque agarose, was ligated into this vector.
25      Enzymes.   Restriction enzymes, T4 DNA ligase and DNA
polymerase I, Klenow fragment were obtained alternatively from
Bethesda Research Laboratories (Gaithersburg, MD), New England
Biolabs (Beverly, MA) or Boehringer-Mannheim (Indianapolis,
IN).

30

B. RESULTS AND DISCUSSION
        Because multiple screenings of previously constructed
human thyroid cDNA library in lambda gt11 (Magnusson, R.P., et
al., Mol. Endocrinol. 1:856-861 (1987)) only yielded

fragments of TPO cDNA, a new thyroid cDNA library in lambda-
Zap was constructed as described herein. The plasmid pHTPO-8S
containing full-length human TPO cDNA was obtained from this
library.  pHTPO-ECE was constructed from pHTPO-8S and the
5    mammalian expression vector pECE (Ellis, L., _et al._, _Cell_
_45_:721-732 (1986)) according to the strategy shown in Figure
1, and was used for subsequent cell transfections.

Chinese  hamster  ovary  cells  were  co-transfected with
pHTPO-ECE and pSV2-neo, and 12 clones were tested for the
10   presence  of  TPO  mRNA  by  northern  blot  analysis.   Total
cellular  RNA  (15  μg/lane)  from  four  pHTPO-ECE transfected
cell lines (CHO-HTPO4, CHO-HTPO12, CHO-HTPO14 and CHO-HTPO17),
and one control pSV2-neo-transfected cell line (CHO-pSV2-neo),
was  subjected  to  northern  blot  analysis  using a human TPO
15   cDNA probe, as described herein.  For comparison, 1 μg of poly
A+ mRNA prepared from a human thyroid gland from a patient
with Graves' disease was used.   28S and 18S ribosomal RNA
markers,  and  an  RNA  molecular  weight  ladder  (B.R.L.,
Gaithersburg,  MD)  were  employed  for  molecular  weight
20   determination.

Four of these clones, as well as one of four control
(pSV2-neo alone) clones, revealed a 3.3 kb mRNA band in the
pHTPO-ECE-transfected clones.  The size of the human TPO mRNA
in the transfected CHO cells is slightly larger than that in
25   the Graves' thyroid cells (3.1 kb), presumably because of the
additional SV40 poly-A coding region at the 3' end of human
TPO cDNA in the pHTPO-ECE plasmid (see Figure 1).

Western  blot  analysis  (under  reducing  conditions)  of
proteins  extracted  from  TPO-transfected  CHO  cells,  using a
30   mouse  monoclonal  anti-human  thyroid  microsomal  antibody
(Portmann, L., _et al._, _J. Clin. Invest._ _81_:1217-1224 (1988)),
revealed an immunoreactive protein of 105-110 kD, as expected
for human thyroid peroxidase (Czarnocka, B., _FEBS Letters_
_109_:147-152 (1985); Ohtaki, S., _et al._, _J. Clin. Endocrinol._

Metab. 63:570-576 (1986)). Briefly, 50 μg of membrane protein
or 30 μg of deoxycholate (DOC)-extracted protein from pHTPO-
ECE-transfected cell lines (CHO-HTPO4, CHO-HTPO12, CHO-HTPO14,
CHO-HTPO17), from a control cell line co-transfected with pECE
5      and pSV2-neo, and from another control cell line transfected
with pSV2-neo alone, were subjected to SDS polyacrylamide gel
electrophoresis under reducing conditions. The proteins were
electrotransferred to nitrocellulose membranes and then
probed, as described herein, with a mouse mAb against the
10      thyroid microsomal antigen (Portmann, L., et al., J. Clin.
Invest. 81:1217-1224 (1988)).

     Strong TPO enzymatic activity was evident in clone CHO-
HTPO12, and in subclones CHO-TPO12b and CHO-TPO12g, obtained
by limiting dilution (Table I). Less enzymatic activity was
15      detected in the other clones. TPO activity in the CHO-TPO12
clones was approximately the same as TPO activity in TSH-
stimulated Graves' thyroid cells in monolayer culture (Table
I).

     In order to determine whether, as with native TPO in
20      thyroid cells, the recombinant, human TPO was expressed on the
surface of the CHO cells transfected with this gene, CHO-
HTPO12b cells were subjected to FACS analysis (Figure 2).
Incubation of these cells with high-titer MSA Hashimoto's
serum (ELISA value of 1.772; normal < 0.2) (Jansson, R., et
25      al., Clin. Exp. Immunol. 63:80-86 (1986)) yielded
approximately 100-fold greater fluorescence than when these
cells were incubated with control serum (Figure 2). Similar
results were obtained with three different Hashimoto's sera.
The size of both the control and Hashimoto's serum-incubated
30      cells was the same (Figures 3E and 3F), excluding the
possibility that differences in cell size were, in part,
responsible for the differences in signal.

     A series of western blot studies was then performed with
protein from CHO-TPO12b cells using a panel of Hashimoto's

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sera with known antimicrosomal antibody levels as determined
by ELISA (Jansson, R., et al., Clin. Exp. Immunol. 63:80-86
(1986)).  Under non-reducing conditions, all 29 Hashimoto's
sera tested, unlike three normal sera, reacted with a major,
5    broad protein band of approximately 200 kD as well as with a
fainter doublet of about 110 kD.  In aggregate, in studies
performed under non-reducing conditions, a total of 36
Hashimoto's sera tested, but not the six control sera, reacted
with these bands.  The interexperimental variability in the
10   intensity of these bands, however, as well as methodological
limitations  in  analyzing  many  samples  simultaneously,
precluded comparison of results of all samples tested.

     Nevertheless, it was apparent that, within a single large
experiment,  the  strongest  signals  were  seen  with  sera
15   containing the highest antimicrosomal antibody ELISA values.
Some sera also recognized protein bands other than those
expected for TPO.  These bands represented wild-type CHO
antigens (presented below).  One apparent TPO-specific signal
of 110 kD also was a non-specific wild-type CHO signal.  This
20   is discussed in more detail below.

     Comparison of the recombinant TPO signals on western
blots performed under reducing and non-reducing conditions
(using  β-mercaptoethanol)  revealed  the  following  with
reduction: (a) loss of the 200 kD broad band; (b) alteration
25   of the 110 kD signal so that it no longer clearly represents a
doublet; and (c) lessening of the specific signals so that
some of the weaker sera become negative.  A non-immune serum
described above that reacted with a band of approximately 110
kD represents a wild-type CHO protein, and not TPO.

30        The specificity of the 200 kD and 110 kD bands discussed
above was demonstrated in two separate experiments utilizing
wild-type,  non-TPO-transfected,  CHO  cells.   In  the  first
experiment, selected, potent Hashimoto's sera tested under the
most favorable (i.e., non-reducing) conditions failed to react

with protein bands of 200 kD or 110 kD.  The second experiment
indicated that the non-immune serum previously shown to react
with a band of 110 kD is a false-positive.  This signal in
wild-type CHO cells is strong despite the use of unfavorable
5       (i.e., reducing) conditions.

        To assess the sensitivity of detection of the specific
signal, western blot analyses were performed with serial
dilutions of two Hashimoto's sera.    The amount of TPO
generated in CHO-TPO12b cells was sufficient to be detected
10      even when these Hashimoto's sera were diluted greater than
3000-fold.

        Human TPO contains 5 potential glycosylation sites.  It
was therefore examined whether carbohydrate moieties are
important in the conformation of the epitope(s) in the human
15      TPO antigen(s) recognized by Hashimoto's sera.  Western blot
analyses were performed on proteins extracted from CHO-TPO12b
cells pre-cultured for 20 hours in 0.5 μg/ml tunicamycin, an
inhibitor of protein glycosylation.  This length of time was
chosen because it was the longest tolerated without evidence
20      of significant toxicity (i.e., cell loss).    Tunicamycin
treatment had no apparent effect on antigen recognition,
suggesting that carbohydrate moieties may not be important
components of the microsomal antigen epitope(s).  In a control
experiment, tunicamycin treatment under similar conditions
25      decreased radiolabeled D-glucosamine incorporation into
proteins by $56.3 \pm 4.8$ % (mean $\pm$ S.D.; n=3).

        An ELISA carried out using antibodies directed against
the microsomal antigen (MSA) was compared with an ELISA
performed with antibodies directed against the recombinant
30      human TPO of the present invention (Figure 3).   Very good
correlation (0.8385249) was observed.  In fact, the anti-MSA
based ELISA resulted in false positives (indicated as
"outlyers" in Figure 3), which were not observed in the ELISA
based upon the anti-recombinant human TPO antibody.

These false positives are likely to result from non-
specific reactions of antithyroglobulin antibodies with the
microsomes, and were not included in the linear regression
calculation for Figure 3.   Support for this conclusion is
5       found in Figure 4, which shows a linear regression analysis
analogous to that shown in Figure 3, but at a much greater
(1/1000) dilution.   It can be seen from Figure 4 that the
increased dilution factor has substantially eliminated the
outlying data points seen at the lower dilution, and that the
10      correlation (0.9060773) is significantly greater. This result
strongly suggests that the lowered specificity of the anti-MSA
based ELISA is, indeed, a function of antigen contamination.
Such problems, which lower assay specificity, might be
addressed by the use of non-recombinant, affinity-purified
15      TPO.   However, generation of truly pure, affinity-purified
natural TPO has proven to be very difficult, if not
impossible, to achieve. These problems are avoided by use of
the recombinant human TPO antigen of the present invention.

In order to further examine its specificity, recombinant
20      human TPO was compared with Graves' thyroid microsomes as a
source of antigen in an ELISA procedure.  The recombinant hTPO
was present in microsomes prepared from a non-thyroidal, non-
human eukaryotic cell line which cannot, therefore, contain
thyroid-specific antigens other than hTPO.     Nevertheless,
25      because sera from patients with autoimmune thyroiditis contain
antibodies against numerous antigens, some of which may be
present in Chinese hamster ovary (CHO) cells (Kaufman, K.D.,
et al., J. Clin. Invest. 84:394-403 (1989)), each serum
sample was also assayed against microsomes prepared from
30      control, non-transfected CHO cells.

In comparing the 51 sera at a standard (1/100) dilution
in both the recombinant hTPO and the thyroid microsomal assay,
a moderately good correlation was observed (r=0.668; p<0.001)
(Fig. 10A).    Clearly, however, there were some widely

discrepant values. In particular, two sera (sera #11 and 27,
Fig. 10A, large circle and square, respectively) that were
very potent in the anti-MSA assay gave values in the anti-hTPO
antibody assay similar to the four normal sera (Fig. 10A, four
5    data points within rectangle near the origin). A number of
other sera, primarily in the high range of activity, also gave
significantly higher values with the thyroid microsomal
preparation than with recombinant hTPO (Fig. 10A). At the
same serum dilution, a much lower correlation was observed
10   between the values obtained with thyroglobulin and recombinant
hTPO as antigen (r=0.315; p<0.05).

     In an autoimmune serum containing antibodies against
multiple antigens, the different antibodies are likely to have
varying affinities for their respective antigens. Serial
15   dilutions of sera will yield different profiles of ELISA
values based on the affinity of each antibody-antigen
interaction. If hTPO is the primary autoantigen in the thyroid
microsomal preparation, the same serum dilution curve should
therefore be observed in assays using thyroid microsomes and
20   recombinant hTPO. In support of this hypothesis, at serum
dilutions of 1/1000 or 1/10,000, the correlation in ELISA
values between thyroid microsomes and human TPO was much
greater (r=0.906 and 0.902, respectively; p<0.001) (Figs. 10B
and 10C). Dramatically, the two sera that were strongly
25   positive with the thyroid microsomal but not with the
recombinant hTPO antigen (Fig. 10A) were no longer
significantly discrepant between the two assays (Fig. 10B and
10C). The dilution curves for these two sera were quite
different in the anti-MSA and anti-hTPO antibody assays (Fig.
30   11A and 11B), confirming that these sera were reacting with
low affinity to an antigen other than hTPO. These two sera
were also distinguished by their surprisingly high levels of
anti-thyroglobulin antibody. In contrast, other sera with
similar anti-MSA levels (at 1/100 serum dilution) yield normal

dilution curves in both assays (sera #12 and 28, Fig. 11A and
11B).

The anti-hTPO antibody ELISA data were also expressed as
the difference between values obtained using the CHO-HTPO
microsomes and the CHO-K1 microsomes as antigen, to correct
for possible interference by anti-CHO cell antibodies
(Kaufman, K.D., et al., J. Clin. Invest. 84:394-403 (1989)).
No significant change was found in the correlation between the
thyroid microsomal and the recombinant hTPO assays using these
revised data at each of the three serum dilutions. Anti-CHO-
K1 antibody ELISA values for the 47 sera of patients with
autoimmune thyroid disease tested, at standard (1/100)
dilution, were 0.164 ± 0.066 SD (mean ± SD).

The precision of the anti-hTPO antibody ELISA was
assessed using three sera chosen to represent a spectrum of
autoantibody potency. Intra-assay variability (10 iterations
for each serum) at standard (1/100) serum dilution, expressed
as mean ± SD (Fig. 12), was 0.346 ± 0.18 (low-potency serum),
0.599 ± 0.44 (medium-potency serum), and 0.923 ± 0.94 (high-
potency serum). The intra-assay coefficients of variation
(CV) for these sera were 5.12%, 7.39%, and 10.2%,
respectively. The inter-assay CV's (7 iterations for each
serum) were 5.36%, 7.63%, and 7.29%, respectively.

In another aspect of the present invention, it has
surprisingly been discovered that CHO cell expression of human
TPO can be significantly increased by employing a different
plasmid. A dihydrofolate reductase (DHFR)-TPO construct has
been made in which both genes (DHFR and TPO) are driven by the
SV40 promoter (Figure 4). Screening of CHO cells transfected
with these constructs has produced two plasmids, designated
pHTPO-DHFR-2B and pHTPO-DHFR-4C, which presently express
three-fold more antigen than that achieved using the pECE-HTPO
plasmid.

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The relative TPO activities observed in CHO cells transfected with pECE-HTPO, pHTPO-DHFR-2B and pHTPO-DHFR-4C are shown plotted against methotrexate concentration in Figure 5. Further, one particular subclone, designated pDHFR-TPO-4C-MTX, has been found to express relatively greater amounts of TPO than any other construct so far isolated, and, in this regard, comprises the best mode presently contemplated of expressing human TPO in CHO cells. The plasmid pDHFR-TPO-4C-MTX was deposited at the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland 20852 on October 3, 1989, with accession number CRL 10250.

Figure 5 shows that, with increasing methotrexate concentrations, a plateau is reached for CHO expression of TPO by the pHTPO-DHFR-2B and pHTPO-DHFR-4C plasmids. While not intending to be bound by any particular theory, one possible explanation for this observation is that the expressed full length TPO gene is toxic to the host CHO cells, resulting in selection for DHFR, but against TPO, at higher methotrexate concentrations. The result of such selection might be that DHFR is amplified while TPO is deleted.

Since the full length TPO gene is membrane-associated, the present inventor hypothesized that it may be possible to increase TPO production in CHO cells if the expressed protein could somehow be dissociated from the membrane. Accordingly, experiments have been undertaken to generate a secretable form of human TPO, by identifying and eliminating the wild-type transmembrane sequence from the gene.

Premature termination in the synthesis of hTPO was hypothesized to reduce the size of the hTPO-M1 protein from 933 to 848 amino acids. An original full length human TPO cDNA clone in Bluescript (pHTPO-BS) was submitted to site-directed mutagenesis to produce plasmid pHTPO(M1)-BS. A single-stranded DNA template was generated, and the indicated 52-mer oligonucleotide probe used for mutagenesis. The

mutations incorporated two stop codons, as well as an EcoRI
site for confirmation, in the region immediately upstream from
the transmembrane region of the human TPO gene (Figure 6).
The entire full length human TPO gene sequence is shown for
5    comparison in Figure 7.

As a consequence of the mutation, a "truncated" human TPO
protein is expressed which is secreted by the host cell rather
than bound to its membrane. The mutated hTPO gene was excised
from pHTPO(M1)-BS using Not I (blunted with Klenow polymerase)
10   and Xba I, and was inserted into the corresponding sites of
pECE-SV2-DHFR, to produce the expression plasmid pHTPO(M1)-
ECE-SV2-DHFR (Figure 8). CHO cells transfected with this
plasmid appear to produce a truncated human TPO protein, which
is believed to retain the antigenic properties of the full
15   length protein, and which, accordingly, comprises another
embodiment of the present invention. Construction of the
plasmid pHTPO(M1)-ECE-SV2-DHFR is summarized in Figure 9.

After stable transfection of CHO cells with the plasmid
pHTPO(M1)-ECE-SV2-DHFR containing the mutated hTPO cDNA,
20   individual colonies of cells (CHO-TPO-M1) were studied for the
expression of TPO (Fig. 14).

Because the kinetics of potentially-secreted hTPO-M1
protein were unknown, the expression of this protein was
initially screened for in CHO cell lysates, since particulate
25   TPO would be expected to be detectable even if the protein
were, in large part, secreted. Randomly selected CHO-TPO-M1
clones showed evidence of variable cellular TPO expression
(Fig. 14). A doublet of approximately 105-101 kD was
specifically immunoprecipitated from lysates of these clones
30   by serum from a patient with Hashimoto's thyroiditis. In CHO
cells transfected with wild-type hTPO cDNA, Hashimoto's serum
immunoprecipitated a doublet of larger size, 112-105 kD, and
neither doublet was detected in non-transfected CHO cells
(Fig. 14A), as previously observed (Kaufman, K.D., et al., J.

Clin. Invest. 84:394-403 (1989)). Immunoreactive TPO was absent from the cell surface of the CHO-TPO-M1 cells, as demonstrated by the lack of immunofluorescence when these cells were pre-incubated with Hashimoto's thyroiditis serum
5    and fluorescently-tagged goat anti-human IgG antibody, unlike CHO cells transfected with wild-type hTPO (Kaufman, K.D., et al., J. Clin. Invest. 84:394-403 (1989)).

In order to determine whether mutated hTPO-M1 is a secreted protein, the biosynthesis and processing of both
10   hTPO-M1 and wild-type hTPO was examined in pulse-chase experiments. First, clone CHO-TPO-M1-K, with the highest expression of truncated TPO (Fig. 14A), was subcloned by limiting dilution, and one cell line (CHO-TPO-M1-K1) was selected for further studies (Fig. 14B). Over a 24 hour chase
15   period, radiolabeled hTPO-M1 protein was secreted by cells into the culture medium and detected by immunoprecipitation with Hashimoto's serum (Fig. 15). This secreted protein was present in the culture medium after 4 hours of chase, with levels accumulating progressively over a 24 hour period.
20   Interestingly, the secreted, immunoprecipitable hTPO-M1 protein appeared as a single band of lesser electrophoretic mobility on the polyacrylamide gel, as compared with its cell-associated form. In contrast, CHO cells expressing wild-type hTPO secreted no detectable immunoprecipitable material into
25   the culture medium. The cell-associated hTPO and hTPO-M1 proteins were similarly stable, with their radiolabeled immunoprecipitates increasing between 0 and 4 hours of chase. Amounts of radiolabeled, immunoprecipitable wild-type hTPO protein at 24 hours of chase were similar to baseline (0
30   hours). The observed decrease in signal in CHO-TPO-M1-K1 cell lysates from 4 to 24 hours is paralleled by an increase in signal in the medium of these cells, supporting the concept of a secreted protein, which, accordingly, comprises another embodiment of the present invention.

In order to prove that the immunoprecipitable material released into the culture media by CHO-TPO-M1-K1 cells was, indeed, TPO, conditioned media were tested for TPO enzymatic activity.    TPO activity (1.0 guaiacol U/10 ml medium) was clearly present in the culture medium from the CHO cells expressing the mutated form of hTPO (Fig. 16).  In contrast, there was no detectable enzymatic activity in conditioned media from CHO cells expressing wild-type hTPO (Fig. 16), despite strong TPO activity present in lysates of these cells, as previously described (Kaufman, K.D., _et al., J. Clin._ _Invest._ **84**:394-403 (1989)).

### Table I

Thyroid Peroxidase (TPO) Activity in CHO-TPO12 Cells
and in TSH-Stimulated Graves' Disease
Human Thyroid Cell Primary Cultures

| Cell Type | Guaiacol Peroxidase (units/mg protein) | Iodide Peroxidase (units/mg protein) |
|---|---|---|
| CHO-pECE (control) | 0 | 0 |
| CHO-pSV2-neo (control) | 0 | 0 |
| Human thyroid cells | 4.7<br>4.6 | 3.0<br>3.4 |
| CHO-HTPO12<br>CHO-HTPO12b<br>CHO-HTPO12g | 3.6<br>4.0<br>3.1 | nd<br>3.1<br>1.9 |

Summary of data from multiple determinations of guaiacol and iodide TPO activity measured in deoxycholate extracts, prepared from 100 mm diameter dishes of the indicated cells. Graves' disease-affected human thyroid cells were cultured for 3 days in 12.5 mU/ml human TSH.
nd - not done

## EXAMPLE XII

### TPO Specific T Cells Infiltrate Thyroid in Graves' Disease

5          Taking advantage of the availability of recombinant TPO,
the occurrence of in vivo selection for T cells specific for
this autoantigen in the intrathyroidal population has been
examined.

10    A.    METHODS
          Infiltrating mononuclear cells were extracted from the
thyroidectomy specimen of a 26 year old female (CX81:HLA-A1,
2; B8, 37; DR3; DRw52; DQw2) with persistently relapsing
Graves' disease and a high titer of antithyroid microsomal
15    antibodies (1:640) by enzyme digestion followed by overnight
incubation and separation of the non-adherent cells as
previously described (Londei, M. et al., Science 228:85-89
(1985)).  The activated cells were selectively expanded by
growth in recombinant IL-2 (Ajinomoto - 20 ng/ml) and 10%
20    human serum in RPMl-1640 (Gibco) for one week.  Cells were
further expanded, nonspecifically with the addition of
irradiated autologous peripheral blood lymphocytes as feeder
cells, OKT3 monoclonal antibody (30 ng/ml) and IL-2 for two
weeks prior to cloning at limited dilution (0.5 cells/well)
25    with OKT3/IL-2 and DR-matched antigen presenting cells (APC).
Further expansion and maintenance of all clones was by 1-2
weekly restimulations with OKT3/IL-2 and HLA unmatched
irradiated feeder cells.  Cells were assayed at the end of the
feeding cycle and a minimum of 5 days after their last
30    exposure to IL-2.
          Proliferation assays were performed over 3 days in
triplicate microtiter wells.  Irradiated autologous PBL (2 - 5
x 10^4) were added to 10^4 clone T cells in 200 μl of 10% human
serum.  1 μl of neat microsome (protein concentration 5 mg/ml)

was added per well. 1 μCi of [³H]thymidine was added for the
final 6 hours of the assay prior to harvesting onto glass
fiber filters and scintillation counting.

Peripheral blood mononuclear cells purified by sucrose
gradient centrifugation (Lymphoprep - Nycomed) were incubated
at 10⁵ cells per well in microtiter wells containing 200 μl
10% human serum. Control or TPO microsomes in 1-2 μl were
added per well as above. Cultures were incubated for 5-6 days
and pulsed with [³H]thymidine in the last 6-18 hours prior to
harvesting and scintillation counting.

Transfection of CHO cells with the complete cDNA for
Human TPO cloned into the expression vector pECE and the
preparation of cell microsomes from transfected and
untransfected CHO cells was as described above.

B.    RESULTS

    In vivo activated thyroid infiltrating T cells were
selected by growth in recombinant IL-2. The resultant
population was then further expanded non-specifically by
stimulation with anti-Cl83 antibodies (OKT3) in combination
with IL-2. Lines so derived consistently showed a marked
response to autologous thyroid epithelial cells in the
absence of added antigen-presenting cells (APC). For example,
the following levels of T cell stimulation, measured as
incorporation of radiolabeled thymidine, were observed:

            T cells:   51 ± 3 cpm;
            Thyroid epithelial cells (TEC):  62 ± 8 cpm;
            T cells + TEC:   6108 ± 1040 cpm.

    T cell clones were obtained by plating the lines at
limiting dilution (0.5 cells/well) followed by further
expansion with IL-2 and OKT3. In this way, antigen-specific
selection was avoided prior to screening of the clones.

    The complete sequence of human TPO cDNA was cloned into
the mammalian expression vector pECE and transfected into

Chinese Hamster Ovary (CHO) cells as described above. These
transfected cells express high levels of immunoreactive and
enzymatically active TPO.    Microsomes prepared from
transfected CHO cells were found to induce significant

5      proliferation of 5 of 24 clones derived from the
intrathyroidal population (Figure 17A). These cells showed no
response to untransfected CHO microsomes (Figure 17A).

       In contrast, peripheral blood T cells (PBL) from the same
individual, from other Graves' patients, or from normal

10     controls, responded to both transfected and untransfected
preparations (Figure 17B). PBL reactivity to CHO cell derived
proteins is not unexpected as similar reactivity has been
described with other xenogeneic cell extracts (Van Vliet, E.
et al., Europ. J. Immunol. 19:213-216 (1989)).  However, it

15     demonstrates the difference in antigenic repertoire between
thyroid infiltrating and peripheral blood T cells, as at no
time was any response to untransfected CHO microsomes seen
with thyroid-derived T cells (Figure 17A and Table IV).

TABLE II

Sites Of NP Synthetic Peptides In Human TPO

```
   1
     933
   _____

NP-1  111 ---- 131
NP-2  116 ---- 131
NP-3      187 ---- 204
NP-4            234 ---- 250
NP-6                  426 ---- 440
NP-7                        535 ---- 551
NP-8                              669 ---- 686
NP-9                                  693 ---- 716
NP-10                                 724 ---- 739
NP-13                 487 ---- 504
   _____
```

Position of synthetic peptides used to screen T cells in human
TPO sequence. Residues are numbered from the amino-terminus.

TABLE III

Responses of T Cell Clone c43 to NP Peptides

| Peptide | Concentration (μg/μl) | | |
|---------|------|------|------|
|         | 0.1  | 1.0  | 10   |
| NP-1    | 48   | 72   | 52   |
| NP-2    | 85   | 54   | 63   |
| NP-3    | 59   | 62   | 63   |
| NP-4    | 50   | 67   | 62   |
| NP-6    | 65   | 60   | 100  |
| NP-7    | 271  | 6190 | 102  |
| NP-8    | 68   | 85   | 16235 |
| NP-9    | 69   | 52   | 221  |
| NP-10   | 63   | 101  | 80   |
| NP-13   | 38   | 69   | 55   |
|         |      |      | 121  |

Responses (in counts per minute, cpm) of thyroid derived T
cell clone c43 to the panel of synthetic peptides of Table II.
Peptides were used at the concentrations shown. Response of
c43 + autologous feeders alone was 101 ± 16 cpm. S.E.M. of
responses was consistently less than 15% of the mean. The
response of c43 to NP-7 was confirmed in 5 subsequent
experiments with similar results.

TABLE IV

Response of T Cell Clones to TPO Microsomes and to NP-7

| Clone | Antigenic Preparation | | | |
|---|---|---|---|---|
| | Control Microsome | TPO Microsome | APC | APC+NP-7 (10μg/μl) |
| c25 | 79 ± 13 | 8024 ± 1144 | 123 ± 32 | 78 ± 8 |
| c39 | 175 ± 22 | 13824 ± 1556 | 236 ± 19 | 276 ± 65 |
| c65 | 54 ± 11 | 1203 ± 111 | 70 ± 4 | 64 ± 6 |
| c69 | 78 ± 10 | 3757 ± 517 | 167 ± 4 | 232 ± 89 |
| c103 | 75 ± 12 | 4575 ± 479 | 76 ± 12 | 54 ± 5 |
| c43 | 654 ± 396 | 2121 ± 554 | 82 ± 12 | 17173 ± 1984 |
| c75 | 84 ± 15 | 151 ± 30 | 346 ± 107 | 2544 ± 135 |
| c104 | 44 ± 6 | 260 ± 26 | 68 ± 14 | 5015 ± 747 |
| c105 | 172 ± 30 | 1028 ± 141 | 599 ± 59 | 4455 ± 338 |
| c3 | 71 ± 11 | 78 ± 20 | 75 ± 11 | 60 ± 5 |
| c9 | 72 ± 4 | 452 ± 32 | 71 ± 4 | 258 ± 46 |
| c18 | 63 ± 3 | 62 ± 10 | 49 ± 6 | 51 ± 1 |
| c20 | 126 ± 21 | 704 ± 89 | 102 ± 9 | 106 ± 17 |
| c29 | 121 ± 27 | 156 ± 17 | 107 ± 7 | 86 ± 5 |
| c60 | 197 ± 110 | 345 ± 84 | 310 ± 53 | 536 ± 50 |
| c64 | 50 ± 3 | 160 ± 13 | 86 ± 20 | 228 ± 52 |
| c70 | 76 ± 17 | 138 ± 23 | 93 ± 20 | 154 ± 20 |
| c77 | 61 ± 8 | 645 ± 284 | 94 ± 11 | 242 ± 43 |
| c82 | 1844 ± 143 | 4246 ± 176 | 8318 ± 191 | 6632 ± 252 |
| c83 | 192 ± 44 | 139 ± 26 | 130 ± 13 | 77 ± 7 |
| c94 | 95 ± 8 | 114 ± 17 | 70 ± 6 | 92 ± 8 |
| c95 | 44 ± 6 | 89 ± 9 | 62 ± 25 | 274 ± 48 |
| c98 | 87 ± 9 | 96 ± 14 | 88 ± 8 | 70 ± 5 |
| c100 | 99 ± 15 | 81 ± 10 | 252 ± 10 | 166 ± 8 |

Responses of thyroid-derived T cell clones to TPO microsomes and peptide NP-7. NP-7 (10 μg/ml) or control or TPO microsomes (0.5 to 1 μl) were added per well as indicated. Autologous irradiated PBL or EBV-transformed B cells were used as antigen presenting cells (APC) at an APC:T cell ratio of between 2 and 5. Results are the mean cpm (± S.E.M.) of triplicate wells. Positive results were confirmed in 2 to 7 different experiments.

Clones were further screened using a panel of 10 synthetic peptides based on the TPO sequence, selected using two T cell motif algorithms (Rothbard, J.8., *Ann. Inst. Pasteur* 137E:518-526 (1986); DeLisis, C. *et al.*, *Proc. Natl. Acad. Sci. USA* 82:7048-7052 (1985)) as shown in Table II. Two clones (c43 and c105) which showed only a small response to TPO microsomes (Table IV) showed a specific response to a peptide (NP-7) corresponding to residues 535-551 of TPO (Table III and IV). Two additional clones (c75 and c104), unresponsive to the whole TPO microsome preparation, showed significant responses to NP-7. In contrast, the 5 clones highly reactive to TPO microsomes (c25, c39, c65, c69, c103) did not respond to NP-7 (Table IV). No response to NP-7 was seen with the patients' peripheral blood T cells (PBL alone = 609 ± 190 cpm; PBL + NP-7 (10 µg/ml) = 302 ± 38 cpm).

## C.    DISCUSSION

The lack of recognition of NP-7 by TPO responsive clones suggests the presence of additional T cell epitopes on TPO distinct from NP-7. The observation that clones specific for an epitope derived from the TPO sequence (NP-7) are present at high frequency in the thyroid infiltrate, and yet respond poorly or not at all to whole TPO presented by APC of peripheral blood origin, is noteworthy.

These results provide the first clear evidence in human organ-specific autoimmunity that a significant proportion of activated T cells infiltrating the target tissue recognize an antigenic protein specific to that tissue. This is consistent with the finding of collagen type II-specific T cells in the joint in rheumatoid arthritis (Londei, M. *et al.*, *Proc. Natl. Acad. Sci. USA* 86:636-640 (1989)). These results also define the site of a T cell epitope within TPO (residues 535-551) and provide evidence for the presence of at least two distinct epitopes on a single target molecule in the same individual.

Such information is very important for the design of appropriate peptide-based immunotherapy, as discussed above.

## EXAMPLE XIII

### Molecular Determination of a B Cell Epitope of TPO

To determine precisely, at the amino acid level, the epitopes in human TPO that are recognized by antibodies in the sera of patients with autoimmune thyroid disease, a panel of mAbs generated against natural TPO was studied. The binding of some of these mAbs to TPO was inhibited by patients' sera, and determination of the TPO epitopes recognized by these mAb would, indirectly, define the disease-associated epitope(s).

This panel of 13 mAbs was used to screen a lambda-Zap library constructed to contain, exclusively, 200-500 bp random fragments of TPO cDNA. When expressed as bacterial fusion proteins, 1/6 of the 3.8 x $10^6$ cDNA fragments would express random 66-166 amino acids fragments of TPO.

For screening, binding of murine anti-TPO mAb (1:40 dilution) was detected using peroxidase-conjugated goat anti-mouse immunoglobulin antibody. Positive plaques were revealed with only one of the thirteen mAb tested (mAb-47). MAb-47 bound TPO with high affinity but did not interfere with the enzymatic activity of TPO. Human anti-TPO autoantibodies strongly inhibited the binding of mAb-47 at 1:20 dilution.

The nucleotide sequences of seven randomly selected clones recognized by mAb-47 were determined. All the clones spanned the same region of the TPO cDNA, overlapping in the region of 2180-2171 bp. This region encodes 30 amino acids (at position 698-728) in the TPO protein.

Anti-TPO mAb-47 is unique among 13 mAbs tested in that it recognizes a continuous epitope on TPO. The other mAbs presumably recognize discontinuous epitopes. The competitive binding to TPO of mAb-47 and naturally occurring anti-TPO

autoantibodies suggests that mAb-47 defines a natural,
disease-associated TPO epitope.

To further elucidate the molecular and cellular basis
for the pathogenesis of autoimmune thyroid disease, it will
be very important to identify the sites (epitopes) on TPO
recognized by the anti-TPO antibodies in Hashimoto's
thyroiditis patients. Prior approaches to the examination of
this question have included the use of immunological probes
(polyclonal or monoclonal anti-TPO antisera) (Libert, F., et
al., EMBO J. 6:4193-4196 (1987); Ludgate, M., et al., J. Clin.
Endocrinol. Metab. 68:1091-1096 (1989); Doble, N.D., et al.,
Immunol. 64:23-29 (1988); Ruf, J., et al., Endocrinol.
125:1211-1218 (1989); Laing, P., J. Clin. Lab. Immunol. 19:19-
23 (1986); Kohno, Y., et al., J. Clin. Endocrinol. Metab.
68:766-773 (1989)) and limited proteolytic digestion
(Yokoyama, N., et al., J. Clin. Endocrinol. Metab. 68:766-773
(1989)). By these means, several distinct antibody binding
regions appear to be present in TPO.

However, TPO is an extremely large antigen
(approximately 107 kD), and these techniques have not allowed
definition of the precise epitopes involved. The present
inventor therefore undertook to screen, with sera from
patients with Hashimoto's thyroiditis, a bacteriophage
(lambda-Zap) human thyroid cDNA expression library containing
large numbers of hTPO cDNA fragments.

Each of these fragments is 200-500 b.p. in length,
coding for TPO polypeptides of 66-166 amino acids. The
entire hTPO protein comprises 933 amino acids. These TPO
polypeptide fragments are expressed as bacterial fusion
proteins, so called because the protein is a hybrid of a 10
kD fragment of β-galactosidase linked to the thyroid protein
component.

METHODS

TPO cDNA fragment library construction: A full-length (3.05 kb) cDNA clone as described above for human thyroid peroxidase was released from its Bluescript vector (Stratagene, San Diego, CA.) by digestion with EcoRI (BRL Laboratories, Gaithersburg, MD) and NotI (Boehringer, Mannheim, West Germany). Because both vector and insert are of similar length, the Bluescript was further digested with ScaI (New England Biolabs, Beverly, MA.). The TPO cDNA was purified by agarose gel electrophoresis and electroelution. The cDNA was then digested (6 minutes at room temperature) into small random-sized fragments with DNAase I (0.1 ng DNase/ug cDNA) (BRL) in 20 mM Tris-HCl, pH 7.5, 1.5 mM $MnCl_2$ and bovine serum albumin, 100 ug/ml. After electrophoresis in 2% SeaPlaque agarose (FMC Bio Products, Rockland, ME), TPO cDNA fragments 200-500 b.p. in length were recovered by electroelution. The ends of the fragments were blunted with the Klenow fragment of DNA polymerase I, and ligated to EcoRI linkers (GAATTCGGCACGAG) containing a nonphosphorylated EcoRI cohesive end and a phosphorylated blunt end (Pharmacia, Piscataway, NJ). After phosphorylation with polynucleotide kinase, excess linkers were removed by electrophoresis in 2% SeaPlaque agarose. The linker-ligated cDNA was again size-selected (200-500 b.p.), electroeluted, ethanol precipitated and ligated into EcoRI-cut lambda-Zap vector (Stratagene). After packaging (Giga-Pak Gold, Stratagene), the library was amplified in XL1-blue cells (Stratagene). cDNA insert sizes were confirmed by the polymerase chain reaction (PCR) (Saiki, R.K., et al., Science 239:487-491 (1988)) using the Bluescript reverse and -20 primers. PCR analysis of the "C2" hTPO cDNA region (Libert, F., et al., EMBO J. 6:4193-4196 (1987); Ludgate, M., et al., J. Clin. Endocrinol. Metab. 68:1091-1096 (1989)) in the TPO cDNA fragment library was performed using two oligonucleotide 22-mer primers (5'-

GGTTACAATGAGTGGAGGGAGT and 5' - GTGGCTGTTCTCCCACCAAAAC)
spanning the region 1852 -2112 b.p. in hTPO (17).  PCR (30
cycles) was for 1 minute at 94°C, 2 minutes at 55°C and 1
minute at 72°C.  For screening the library, the PCR-generated
DNA was labeled with $^{32}P$-$\alpha$CTP to a specific radioactivity of
0.8 x $10^9$ cpm/vg DNA using the random primer method
(Multiprime; Amersham, Arlington Heights, IL).  The screening
procedure employed standard techniques (Maniatis, T., _et al.,_
_Molecular Biology:  A Laboratory Manual,_ Cold Spring Harbor
Laboratory, Cold Spring Harbor, NY (1982)), with final washes
of 30 minutes (x 2) at 55°C in 0.1 x SSC, 1% SDS buffer (1 x
SSC in 150 NaCl, 15 mM Na citrate, pH 7.5).  Autoradiography
of the nitrocellulose filters was performed with Kodak XAR-5
film.

_Immunological screening of the TPO sub-library:_  The lambda-
Zap library containing TPO cDNA fragments, plated in _E. coli_
Y1090 at about 3 x $10^4$ pfu per 150 mm diameter Petri dish,
was screened as previously described (Seto, P., _et al., J._
_Clin. Invest._ _80_:1205-1208 (1987)).  In brief, after 3.5
hours at 42°C, nitrocellulose filters soaked in 10mM
isopropyl-thio-beta-D-galactopyranoside (IPTG) were overlayed
for 3.5 hours at 37°C.  Filters were removed, washed in TBS
buffer (10 mM Tris HCl, pH 7.5, 150 mM NaCl) containing 0.05%
Tween, incubated for 15 minutes at room temperature in
TBS/Tween containing 2% Carnation milk, rinsed with TBS/Tween,
and then incubated overnight at 4°C with antibody.  For
immunological screening, mouse monoclonal antibody (#20.10)
against the thyroid microsomal antigen (Portmann, L., _et al.,_
_J. Clin. Invest._ _81_:1217-1224 (1988)) was used at a 1:200
dilution.  Because of the very low background and strong
signal achieved with monoclonal antibodies, pre-adsorption
with bacterial proteins is not necessary prior to screening,
as previously described (Seto, P., _et al., J. Clin. Invest._

80:1205-1208 (1987)). Antisera from 13 Hashimoto's
thyroiditis patients with high titer antimicrosomal antibodies
were used under a variety of different conditions at a
dilution of 1:200.  In contrast to previous experience in

5   screening lambda gt11 libraries with Hashimoto's sera (Hirayu,
H., et al., J. Clin. Endocrinol. Metab. 64:578-584 (1987)),
screening of the lambda-ZAP libraries provided very little
background with such sera, and, in general, pre-adsorption was
not required to reduce this non-specific background.  When

10  pre-adsorption was performed, Y1090 proteins were immobilized
on nitrocellulose filters.  In addition, affinity-purified
anti-TPO antibodies, prepared using recombinant hTPO expressed
on the surface of Chinese hamster ovary (CHO) cells also were
used as immobilized antigen (Kaufman, K.D., et al., J. Clin.

15  Invest. 84:394-403 (1989)).  For this procedure, 1 ml of
serum was diluted 1:10 in phosphate-buffered saline (PBS)
containing  0.05%  Na  azide  and  1mM  phenylmethyl
sulfonylfluoride (PMSF).  TPO-CHO cells (approximately $10^8$)
were resuspended by light trypsinization, diluted in PBS

20  containing 10% calf serum, pelleted (5 minutes at 1,000 x g),
and resuspended in the diluted antibody for 1 hour at 4°C.
Unbound antibody was removed by pelleting the cells, followed
by a rinse in ice-cold PBS. After recovery by centrifugation
(5 minutes at 1,000 x g), the cells were resuspended and

25  incubated for 15 minutes at 4°C in 150 mM acetic acid in PBS
containing 0.05% Na azide and 1 mM PMSF.  NaOH and 1 M Tris,
pH 7.5, were added to neutralize the acetic acid, and the
cells and particulate material were removed by centrifugation
(5 minutes at 1,000 x g, and then for 30 minutes at 100,000 x

30  g, 4°C), leaving the affinity-purified antibody in the
supernatant.  The efficiency of the affinity purification was
approximately 50%, as measured by ELISA (Schardt, C.W., et
al., J. Immunol. Methods 55:155-168 (1982)).

The detection systems for antibody bound to fusion
proteins were as previously described (Seto, P., et al., J.
Clin. Invest. 80:1205-1208 (1987)), using the following
antisera:  For the mouse antimicrosomal monoclonal antibody,
peroxidase-conjugated, affinity-purified goat anti-mouse IgG
(heavy and light chain specific) (Cappel, Organon, West
Chester, PA.) at a dilution of 1:300; For the polyclonal
human antisera, anti-human IgG (Fc fragment, gamma chain
specific) (Cappel) at a dilution of 1:300.  Color was
developed with 2.8 mM 4-chloro-1-naphthol (Sigma, St. Louis,
MO).  The quality of the immunological reagents used in the
polyclonal antibody screening procedure was confirmed by
their ability to generate a strong signal with eukaryotic
recombinant hTPO on western blot analysis (Kaufman, K.D., et
al., J. Clin. Invest. 84:394-403 (1989)).  Positive clones
were plaque-purified to homogeneity.  Control screening of
potentially positive plaques was performed by omitting the
first (anti-TPO) antibody in the screening procedure.

Nucleotide sequence analysis of selected clones:  Plaque-
purified lambda-Zap phage were used to generate Bluescript
plasmids containing the fragment of TPO cDNA whose respective
fusion proteins had been detected by the antisera.  This
procedure used the helper phage R408 according to the
protocol of the manufacturer (Stratagene).  Rescued phagemids
were used to infect XL1-blue bacteria (Stratagene).  Plasmids
were prepared from individual colonies (Maniatis, T., et al.,
Molecular Biology: A Laboratory Manual, Cold Spring Harbor
Laboratory, Cold Spring Harbor, NY (1982)), and the sizes of
the cDNA inserts were assessed by digestion with EcoRI.
Nucleotide sequencing of selected plasmid cDNA inserts was
performed by the dideoxynucleotide termination method (Sanger,
F., et al., Proc. Natl. Acad. Sci. USA 74:5463-5467 (1977)).

Nucleotide sequence analysis was performed using the software provided by Bionet.

RESULTS

Localization of the epitope for a monoclonal antibody against thyroid peroxidase. In order to define the epitope(s) for anti-TPO antibodies in patients with autoimmune thyroid disease, it was first necessary to determine the validity of the immunological screening of a hTPO cDNA fragment sub-library (Mehra, V., et al., Proc. Natl. Acad. Sci. USA 83:7013-7017 (1986)). For this purpose, a monoclonal antibody generated against the thyroid microsomal antigen (Portmann, L., et al., J. Clin. Invest. 81:1217-1224 (1988)) that had been used successfully in the past to clone this antigen from a Graves' thyroid cDNA library (Hirayu, H., et al., J. Clin. Endocrinol. Metab. 64:578-584 (1987)) was used. The new TPO cDNA fragment sublibrary constructed contained 3.8 x 10⁶ recombinant clones, with an effective (correct orientation and reading frame) size one-sixth of this number. The insert sizes were confirmed to be in the 200-500 b.p. range.

Screening of this library with the anti-microsomal antigen monoclonal antibody yielded 6-12 positive plaques per 1,000 plaques screened. Fourteen positive clones were randomly chosen for partial nucleotide sequencing to delineate the position of their TPO cDNA inserts relative to the entire TPO gene. Twelve of the 14 clones had cDNA inserts of 160-350 b.p. Two clones (U and Y) that had cDNA inserts slightly larger than the expected 500 b.p. maximum were found, upon nucleotide sequencing, to have double cDNA inserts. As an indication of the success of the procedure, all 14 clones recognized by the monoclonal antibody spanned the same region (746-1,150 b.p.) of the hTPO gene (Magnusson, R.P., et al., Mol. Endocrinol. 1:856-861 (1987)) (Figure 18). The maximum region common to all clones, and therefore an

indication of a common epitope, was between bases 881 and 927
(AA AAC CCA TGT TTT CCC ATA CAA CTC CCG GAG GAG GCC CGG CCG
GCC), corresponding to a derived amino acid sequence of only
15 residues (Asn Pro Cys Phe Pro Ile Gln Leu Pro Glu Glu Ala
5      Arg Pro Ala).   Therefore, the epitope recognized by the
monoclonal antibody lies within this 15 amino acid span.

Epitope(s) for the antimicrosomal/TPO antibodies in
autoimmune thyroid disease.  Approximately forty screenings
of the same TPO cDNA fragment sub-library described above
10     with sera from patients with Hashimoto's thyroiditis did not
yield any positive clones. The modifications that were tried
included: 1) the use of different host bacteria (BB4, XL1
blue and Y1090) in which to express the TPO fusion proteins;
2) variation in the antibody binding detection system,
15     including the use of anti-human IgG antibody or protein A
from different vendors, as well as different incubation times
and temperatures; and 3) the use of thirteen different
patients' sera with potent anti-TPO activity. The sera were
tested in multiple ways: without bacterial pre-adsorption;
20     following adsorption with bacterial lysate; or after
affinity-purification with recombinant hTPO.   As internal
controls in the screening procedure, the monoclonal antibody
always yielded the expected number of positive clones.

Quite surprisingly, it was not possible to detect the
25     epitope expressed within the 86 amino acid C2 hTPO
polypeptide fragment, as previously reported (Libert, F., et
al., EMBO J. 6:4193-4196 (1987); Ludgate, M., et al., J. Clin.
Endocrinol. Metab. 68:1091-1096 (1989)).   Because of the
possibility that the fragment library employed might lack the
30     C2 region, C2 region presence was tested by PCR, using
oligonucleotide primers complementary to each end of the C2
region.   A fragment of the expected size (261 b.p.) was
clearly detected.   Further, by using this PCR-generated
fragment as a probe to screen the library, it was determined

that approximately 10% of the plaques in the library contain
C2 sequence.

Because of these negative results with the Hashimoto's
thyroiditis sera in the hTPO cDNA fragment library, these
sera also were used to screen lambda-Zap Graves' thyroid
libraries (both oligo-dT and random-primed), constructed as
described previously (Kaufman, K.D., et al., J. Clin. Invest.
84:394-403 (1989)).   The oligo-dT-primed library contains
numerous full-length copies of TPO cDNA (3.1 kb), as was
demonstrated by the ability to express enzymatically active,
antigenically intact TPO, when such cDNA was subcloned from
the phage vector into a eukaryotic expression plasmid, and
stably-transfected into eukaryotic Chinese hamster ovary cells
(Kaufman, K.D., et al., J. Clin. Invest. 84:394-403 (1989)).
Despite this, no specific signal was detected in screening
this lambda-Zap library with 13 potent Hashimoto's sera that
strongly   react   immunologically   with   TPO   expressed   in
eukaryotic cells (Kaufman, K.D., et al., J. Clin. Invest.
84:394-403 (1989)).   Many strongly reacting plaques were
observed in these screenings, in which plaques reacted with
the second antibody (anti-human IgG) even in the absence of
patients' serum.  Similar findings were obtained in the past
with a Graves' thyroid cDNA library in lambda gt11 (Hirayu,
H., et al., J. Clin. Endocrinol. Metab. 64:578-584 (1987)).
These clones may represent IgG present in B-lymphocytes in the
Graves' thyroid gland from which the library was made.

A potential difficulty with protein expression in a
full-length cDNA phage library is that stop codons in the 5'-
untranslated region of the cDNA insert may interrupt the
translation  of  the  foreign  protein,  which  is  inserted
downstream  of  the  $\beta$-galactosidase  portion  of  the  fusion
protein.    To  eliminate  this  possiblity,  two  additional
approaches were attempted.   The first was screening of a
random-primed   human   thyroid   cDNA   lambda-ZAP   library,

constructed in the same manner as the oligo-dT primed
library, with the exception that random primers, rather than
oligo-dT, were used for first strand cDNA synthesis. This
library contains cDNA clones with a bias against full-length

5    cDNA copies. The second approach was to delete the 5'-
untranslated region from the full-length hTPO cDNA clone in
the Bluescript plasmid generated from the lambda-Zap clone
(Kaufman, K.D., et al., J. Clin. Invest. 84:394-403 (1989)).
This deletion was accomplished by digestion of this plasmid

10   with XhoI, thereby releasing 154 b.p. of the 5'-end of hTPO
cDNA, leaving the entire TPO protein (minus the signal
peptide) remaining in reading frame with the β-galactosidase
component of the Bluescript plasmid. This new plasmid
construct was transfected into XL1-Blue host bacteria for

15   fusion protein generation (Stratagene, San Diego CA) and
western blot analysis. Neither the random-primed library nor
the XhoI deletion mutant generated a hTPO protein that could
be recognized by Hashimoto's antisera, or with anti-TPO
antibody affinity-purified from these sera using recombinant

20   hTPO.


DISCUSSION

     The present data provide the first definition, at a
precise molecular level, of an epitope recognized by an

25   antibody against a thyroid autoantigen. Previous studies
using polyclonal or monoclonal antibodies against human
thyroglobulin (Male, D.K., et al., Immunol. 54:419-426 (1985);
Fukuma, N., et al., Immunol. 67:129-131 (1989)) or TPO
(Libert, F., et al., EMBO J. 6:4193-4196 (1987); Ludgate, M.,

30   et al., J. Clin. Endocrinol. Metab. 68:1091-1096 (1989);
Doble, N.D., et al., Immunol. 64:23-29 (1988); ; Laing, P., J.
Clin. Lab. Immunol. 19:19-23 (1986); Kohno, Y., et al., J.
Clin. Endocrinol. Metab. 68:766-773 (1989); Yokoyama, N., et
al., J. Clin. Endocrinol. Metab. 68:766-773 (1989)) have

suggested that these antibodies recognize different regions of
the antigen, but no study has been able to localize an epitope
to a region of the molecule as small as 15 amino acid residues
in size. The minimum size of a B-cell (antibody-recognized)
5      epitope is under discussion, but is believed to be on the
order of 5-10 amino acid residues (Van Regenmortel, M.H.V., et
al., Immunol. Lett. 17:95-108 (1988)). Therefore, the 15
residue span of the present invention is very close to the
size of the epitope itself.

10         A remarkable finding in this example is the striking
contrast between the positive results with the anti-
microsomal/TPO monoclonal antibody, and the inability of
naturally-occurring, disease-associated anti-TPO antibodies
to recognize the 66-166 amino acid TPO fragments expressed in
15     the library employed. Unlike more linear T-cell epitopes,
naturally occurring B-cell epitopes may be more
conformational, and subject to influence by the secondary or
even tertiary structure of the molecule. Disulfide bonds and
contiguity of loops of the folded protein that may be far
20     distant in its linear structure, may contribute to the
formation of a B-cell epitope. The present data suggest that
the epitope(s) for the disease-associated anti-TPO antibodies
are highly conformational.

25                         EXAMPLE XIV

Further Determination of the B Cell Epitope on TPO

       This example provides an important step in understanding
the pathogenesis of Hashimoto's thyroiditis by defining the
30     epitope recognized by antithyroid peroxidase (anti-TPO)
antibodies. In Example XIII, a human TPO cDNA sublibrary was
constructed expressing random fragments of the protein (each
66-166 amino acids in length) (Mehra, V., et al., Proc. Natl.
Acad. Sci. USA 83:7013-7017 (1986)). However, serum from

patients with Hashimoto's disease with high titers of anti-TPO
antibodies failed to recognize any of these TPO protein
fragments.  In contrast, TPO fragments in this library were
recognized by a mouse monoclonal antibody (MAb) against
5      denatured human TPO.  These data support previous evidence
(Hamada, N., et al., J. Clin. Endocrinol. Metab. 64:230-238
(1987); Nakajima, Y., et al., Mol. Cell. Endocrinol. 53:15-23
(1987)) that the disease-associated TPO epitopes are highly
conformational and are likely to be formed by noncontiguous
10     (discontinuous) regions of the linear amino acid sequence.
        This example presents the determination of the disease-
associated 8-cell epitopes on TPO, using a panel of 13 MAb
generated against nondenatured human TPO (Ruf, J., et al., :
Endocrinology 125:1211-8 (1989)).  The binding of some of
15     these MAb to native TPO is inhibited by anti-TPO antibodies in
the serum of patients with autoimmune thyroid disease (Ruf,
J., et al., Endocrinology 125:1211-8 (1989)), indicating that
these particular MAb epitopes correspond to or are in the
vicinity of the disease-associated epitopes.  Determination of
20     the epitopes for some of the TPO MAb in the panel could,
therefore, delineate molecular domains of the autoimmune
thyroid disease-associated B-cell epitopes.


MATERIALS AND METHODS
25

TPO Fragment Library:  The construction of the TPO random
fragment cDNA library (3.8 x $10^6$ plaque-forming units) has
been described previously.  Immunoscreening of the library was
performed by standard techniques, as previously described,
30     using 13 mouse MAb generated against native human TPO (Ruf,
J., et al., Endocrinology 125:1211-8 (1989)).  Positive clones
were plaque-purified and used to generate Bluescript plasmids
for nucleotide sequencing of the cDNA inserts (Sanger, F., et
al., Proc. Natl. Acad. Sci. USA 74:5463-5467 (1977)).  The

DNA sequences of independent clones were aligned with the TPO cDNA sequence to localize the minimum region of overlap that encompasses the epitope (Mehra, V., _et al._, _Proc. Natl. Acad. Sci. USA_ _83_:7013-7017 (1986)).

5      _Western Blots_:   Recombinant human TPO stably expressed by Chinese hamster ovary cells was used as antigen.  Cells were cultured, scraped into buffer containing 10 mM Tris (pH 7.4), 0.25 M sucrose, 2 mg/mL bacitracin, 1 mM phenylmethylsulfonyl-
10     fluoride, 0.1 mM N-α-p-tosyl-L-lysine-chloromethylketone, and 0.1 mM leupeptin (all from Sigma Chemical Co., St. Louis, MO), and a microsomal fraction was prepared, all as previously described.   The protein concentration was determined by the method of Bradford (Bradford, M.M., _Anal. Biochem._ _72_:238-254
15     (1976)).    Samples (~100 μg protein) were treated with 2% sodium  dodecyl  sulfate  and  5% β-mercaptoethanol  (final concentrations)  and  subjected  to  7.5%  polyacrylamide  gel electrophoresis (Laemmli, U.K., _Nature_ _227_:680-685 (1970)). Proteins  were  transferred  to  a  ProBlot  membrane  (Applied
20     Biosystems,  Foster  City,  CA)  using  the  MilliBlot  transfer system  (Millipore  Co.,  Bedford,  MA)  according  to  the manufacturer's recommendations.  Membranes were processed as previously described, with minor modifications.   Incubations with MAb (1:1000 dilution) were performed overnight at 4°C.
25     MAb binding was detected with horseradish peroxidase-linked sheep  antimouse  immunoglobulin  G  F(ab')2  (Amersham International,  Aylesbury,  Buckinghamshire,  United  Kingdom) diluted  1:1,000  using  0.5  mg/mL  4-chloro-1-naphthol,  0.57 mg/mL imidazole, 17% methanol, and 0.42% hydrogen peroxide as
30     substrate.

_RESULTS_

Of the 13 mouse MAbs generated against nondenatured human
TPO (Ruf, J., et al., Endocrinology 125:1211-8 (1989)), only
1 (no. 47) recognized TPO protein fragments expressed by the
cDNA library. The nucleotide sequences were determined for 18
5    randomly selected cDNA clones. All cDNA inserts spanned the
same region of the TPO cDNA sequence (Figure 19). The minimal
region common to all cDNA fragments was between basepairs 2219
and 2247 of the human TPO cDNA nucleotide sequence, coding
for 9 amino acids (residues 713-721) in the protein. These
10   nine amino acids thus represent at least a part of the epitope
for anti-TPO monoclonal antibody 47. The inability of the
other 12 TPO MAb to recognize TPO peptide fragments expressed
by the library could not be attributed to technical
difficulties in the screening procedure, because internal
15   controls, TPO MAb 47 and TPO MAb 20.10 (Portmann, L., et al.,
J. Clin. Invest. 81:1217-1224 (1988)) all were strongly
positive.

To compare the reactivity of the panel of 13 MAb to TPO
fragments generated by the cDNA library (see above) with
20   reactivity to the entire TPO protein, Western blot analyses
were performed using these MAb as probes and recombinant human
TPO expressed in CHO cells as antigen. For the TPO
fragments, only MAb 47 reacted with the entire TPO molecule
under denaturating and reducing conditions (Figure 20). As a
25   control, TPO MAb 20.10 (Portmann, L., et al., J. Clin. Invest.
81:1217-1224 (1988)), generated against the denatured protein
and previously shown to recognize a linear epitope between TPO
amino acids 266-281 (Finke, R., et al.. J. Clin. Endocrinol.
Metab. 71:53-59 (1990)), also detected a protein of similar
30   size. Consistent with previous enzyme-linked immunosorbent
assay data (Ruf, J., et al., Endocrinology 125:1211-8 (1989)),
all 13 MAb against native TPO immunoprecipitated nondenatured
recombinant human TPO.

DISCUSSION

     The present data demonstrate that only one (no. 47) of a
panel of 13 MAb generated against native human TPO reacts with
5    random 66-166 amino acid fragments of the 933-amino acid TPO
molecule.    Consistent with this observation, only MAb 47
recognizes intact TPO after denaturation and reduction,
although all 13 MAb in this panel recognize native
nondenatured human TPO (Ruf, J., et al., Endocrinology
10   125:1211-8 (1989)). In agreement with our findings, MAb 47 is
unique in this panel of TPO Mab, in that it was the only MAb
whose binding to TPO could not be abolished by dithiothreitol
treatment of the protein. The epitope for TPO MAb 47 (amino
acids 713-721) is different from that for TPO MAb 20.10 (amino
15   acids 266-281). Furthermore, TPO MAb 20.10 reacts only with
denatured TPO (Portmann, L., et al., J. Clin. Invest. 81:1217-
1224 (1988)).
     Our findings reinforce the emerging concept that many B-
cell epitopes are conformational and are likely to be
20   discontinuous. By this it is meant that epitopes on globular
proteins are dependent on 3-dimensional structure and consist
of a number of different regions of the linear protein brought
into apposition by protein folding. Thus, only 1 of 13 MAb
generated by immunizing mice with native TPO recognizes a
25   linear epitope expressed in a TPO fragment library or after
unfolding of TPO by denaturation and reduction. Because MAb
47 also recognizes the native TPO protein, amino acids 713-721
must be situated on the surface of human TPO (unlike amino
acids 266-281 recognized by TPO MAb 20.10). Other contiguous
30   loops in the folded protein derived from different regions of
the linear sequence may also contribute to the epitope for MAb
47.   Amino acids 713-721 may be the minimum needed for
recognition by the antibody.

The binding of TPO MAb 47 to human TPO is inhibited by
anti-TPO antibodies in the serum of patients with autoimmune
thyroid disease (Ruf, J., et al., Endocrinology 125:1211-8
(1989)). Therefore, the linear nine-amino acid (residues 713-
5      721) epitope for MAb 47 either corresponds or is close to an
autoantibody-associated TPO B-cell epitope. The present data
define specific amino acids in a domain containing an epitope
for thyroid autoantibodies. Competition studies with MAb 47
(Ruf, J., et al., Endocrinology 125:1211-8 (1989)) suggest
10     that the idiotypic antibody in autoimmune thyroid disease
serum that interacts with the MAb 47 epitope is uncommon.


                          EXAMPLE XV

Overexpression of Secreted hTPO in Non-Thyroidal Eukaryotic
15  Cells

       Previous examples describe expression of recombinant
human TPO (hTPO) as both the native, membrane-associated
enzyme and as a truncated, secreted protein. In the present
example, the overexpression of the secreted form of
20     recombinant hTPO in eukaryotic cells is described. hTPO gene
amplification was accomplished with a vector containing the
mouse dihydrofolate reductase (dhfr) gene. Stably
transfected Chinese hamster ovary (CHO) cells were grown in
the presence of progressively increasing concentrations of
25     methotrexate (MTX). TPO expression was measured
immunologically in an enzyme-linked immunosorbant assay
(ELISA) using anti-TPO antibodies. Attempts to also
overexpress the wild-type, membrane-associated form of the
enzyme were less successful. While some amplification of the
30     native hTPO gene was observed, it was not possible to achieve
a level of protein expression significantly higher than that
observed in some high-producing cell lines prior to
initiation of selective pressure by MTX. Indeed, above 100
nM MTX, the immunoreactive hTPO content of cells actually

diminished.   In contrast, progressive overexpression of the truncated, secreted form of hTPO up to a final MTX concentration of 10,000 nM was observed.  Slot-blot analysis of genomic DNA from transfected cells revealed parallel

5      amplification of the dhfr and truncated hTPO genes.  High-level expression of secreted hTPO provides a means by which large amounts of biologically and immunologically active hTPO protein may be obtained.

10     MATERIALS AND METHODS
       Construction of the expression plasmids pSV2-DHFR-ECE-hTPO and pSV2-DHFR-ECE-hTPO-M1:   Full-length hTPO cDNA in the expression vector pECE was digested with PvuI and the ends blunted with the Klenow fragment of DNA polymerase I.  The

15     expression vector pSV2-dhfr (kindly provided by Dr. Gordon Ringold, Syntex, Palo Alto, CA) was digested with EcoRI, the ends blunted with Klenow fragment of DNA polymerase I, and the vector treated with bacterial alkaline phosphatase.  The blunt-ended, linearized vector and cDNA were ligated together

20     to form the recombinant plasmid pSV2-DHFR-ECE-HTPO.  The cDNA coding for the secreted form of hTPO (hTPO-M1), generated in Bluescript by site-directed mutagenesis, was exchanged for wild-type hTPO cDNA in the plasmid pSV2-DHFR-ECE-HTPO to generate pSV2-DHFR-ECE-HTPO-M1.

25
       Transfection of pSV2-DHFR-ECE-HTPO and pSV2-DHFR-ECE-HTPO-M1 into CHO dhfr- cells and amplification with methotrexate: CHO dhfr- cells (CHO-DG44; kindly provided by Dr. Robert Schimke, Stanford University, Palo Alto, CA) were maintained

30     in Ham's F-12 medium supplemented with 10% fetal calf serum, penicillin (100 U/ml), gentamicin (40 ug/ml) and amphotericin B (2.5 ug/ml).   Transfection with plasmid DNA (10 ug) was performed by the calcium phosphate precipitation method (Chen, C., et al., Mol. Cell. Biol. 7:2745-2752 (1987)).

Transfected cells were selected for in thymidine-, guanidine-,
and hyposanthine-free Ham's F-12 medium supplemented with 10%
dialyzed  fetal  calf  serum  and  antibiotics  as  above.
Individual clones were selected with cloning cylinders and 2
5    clones with high levels of TPO expression (clones CHO-HTPO-2B
and CHO-HTPO-C4C) were subsequently used for amplification.
Methotrexate (MTX) was added to this selective cell culture
medium as an initial concentration of 3.3 nM and surviving
cells  were  harvested  and  expanded.   The  methotrexate
10   concentration  was  sequentially  increased  by  3.33-fold
increments until a final concentration of 10,000 nm (100 μM)
was reached.

ELISA of CHO-hTPO and CHO-hTPO-M1 cells:  ELISA of human sera
15   (kindly provided by Dr. Sandra McLachlan, Cardiff, Wales, UK)
of control and MTX-treated CHO-hTPO cells was modified from
the method of Schardt et al. (J. Immunol. Methods 55:155-168
(1982)),  as  described  above,  using  cellular  microsomes.
Because the hTPO-M1 protein is secreted into the medium of
20   CHO-hTPO-M1 cells, three-day conditioned media were collected
from these cells.  Proteins from these media were precipitated
and treated, as described above.  Antigen for ELISA of human
sera was applied as 100 ul of the dialyzed protein precipitate
per well, approximately 300 ug protein diluted 1:1 in 2 x
25   coating buffer (0.1 M sodium bicarbonate, pH 9.3 + 0.04%
sodium azide).  Because more than one ELISA was used for all
MTX concentrations, values are reported as an ELISA index
referenced to 1000 nM MTX values used across assays of each
cell type.  The same sera were used in ELISAs of each cell
30   type.

Genomic DNA extraction of CHO-hTPO-M1 cells:  Cells from
confluent  100  mm  diameter  dishes  of  CHO-hTPO-M1  cells
surviving at each MTX concentration were frozen and kept at

-80°C until replated (100 mm dish), grown to confluence, and
used for extraction of genomic DNA. Cells were rinsed three
times in 5 ml ice-cold Dulbecco's phosphate-buffered saline,
calcium- and magnesium-free (PBS-CMF). The cells were then
5       scraped from the dish, recovered by centrifugation for 10
minutes at 2000 rpm, 4°C. The pellet was resuspended in 2
volumes (100-200 ul) 320 mM sucrose, 10 mM Tris-Cl, pH 7.5, 5
mM MgCl$_2$, 1% Triton X-100, and kept on ice for 30 minutes.
The suspension was centrifuged for 15 minutes at 2500 rpm
10      (4°C), and the pellet resuspended in 4.5 ml 10 mM NaCl, 10 mM
Tris-Cl, pH 7.5, 10 mM EDTA. RNAse digestion (addition of
4.5 $\mu$l 10 mg/ml DNase-free RNase for 60 min at room
temperature) was followed by proteinase K digestion
overnight at 37°C (addition of 0.5 ml 10% SDS + 0.1 ml 10
15      mg/ml proteinase K). The DNA was then extracted two or three
times (until the aqueous phase was clear) with 5 ml 0.1 M
Tris-buffered phenol, pH 7.4:CHCl$_3$, 4% isoamyl alcohol (1:1),
followed by an equal volume extraction with CHCl$_3$, 4% isoamyl
alcohol. The DNA was precipitated with 0.1 volume 3 M sodium
20      acetate, pH 5.2 and 2 volumes ethanol at -80°C for 2 hours and
the pellet resuspended in 0.5 ml TE (10 mM Tris, pH 8.0, 1 mM
EDTA). Quality and quantity of genomic DNA samples were
assessed by agarose gel electrophoresis and OD at 260 nm.
Genomic DNA yield from a 100 mm dish of confluent cells was
25      40-160 $\mu$g.

Slot blot analysis of CHO-hTPO-M1 cells: Genomic DNA (15 ug)
from CHO-hTPO-M1 cells was digested with EcoRI, ethanol-
precipitated, resuspended in TE buffer, and requantified by
30      OD at 260 nm. Aliquots of this DNA (1.0, 0.5, or 0.25 ug)
were diluted in 0.5 ml 0.4 N NaOH, 10 mM EDTA, boiled for 10
minutes and placed on ice. Nylon membrane filters (Hybond-N
RPN, 3050N, Amersham Corporation, Arlington Heights, IL),
rinsed in 0.4 N NaOH, were applied to a slot-blot apparatus

(Minifold II, Schleicher & Schuell, Keene, NH) and the wells
were rinsed with 0.5 ml 0.4 N NaOH and vacuum dried.
Individual 0.5 ml genomic DNA samples were added per well,
vacuum was applied briefly, and the wells were rinsed with
5   0.5 ml 0.4 N NaOH and vacuum dried.  The filters were
removed, washed briefly in 2x SSC (0.3 M NaCl, 0.03 M sodium
citrate, pH 7.0) and air dried. Genomic DNA was cross-linked
to the filters by UV irradiation (UV Stratalinker 2400,
Stratagene, La Jolla, CA), and the filters probed with a
10  labeled, PCR-derived, 0.3 kb fragment of the mouse dhfr cDNA,
washed, and autoradiograms performed.  Following confirmed
removal of first label after boiling in 0.1x SSC (0.015 M
NaCl, 0.0015 M sodium citrate), 0.1% SDS for 1 hour, the
filters were reprobed with a labeled 0.56 kb fragment of human
15  TPO cDNA, washed, and photographed.

RESULTS
       Recombinant plasmids pSV2-dhfr-ece-hTPO and pSV2-dhfr-
ece-hTPO-M1 were transfected into CHO dhfr- cells to produce
20  CHO-TPO and CHO-TPO-M1 cell lines, respectively.  These cell
lines were grown in progressively increasing (3.33 fold) MTX
concentrations up to 1000 (membrane-associated hTPO) or
10,000 (secreted hTPO), each cycle taking a minimum of three
weeks.   Cells at each stage of amplification were cryo-
25  preserved and were replated after the final amplification
step for comparison of the levels of immunoreactive hTPO
expression.
       Content of wild type membrane-associated human TPO in
microsomal fractions from cell lines CHO-HTPO-25 and CHO-
30  HTPO-C4C was quantitated immunologically by ELISA using anti-
TPO antibodies in Hashimato's thyroiditis serum. In both cell
lines, some degree of amplification of TPO immunoreactivity
was evident with increasing MTX concentrations, reaching a
maximum at 100 nm MTX.  This increase was followed by a

gradual fall in immunoreactive TPO protein at higher MTX
concentrations up to 1 uM. There was a minimal increase of
TPO expression in CHO-HTPO-C4C, the cell line with the higher
basal (pre-MTX) hTPO content. While there was a greater

5   increment in TPO expression in CHO-HTPO-2B cells, the maximum
level achieved was only slightly higher than that in the CHO-
HTPO-C4C cells. During MTX-induced gene amplification of both
the CHO-HTPO and CHO-HTPO-M1 cells, there appeared to be
greater cell death at the 100 to 333 nM MTX step than at lower

10  concentrations, with a delay in growth of surviving cells to
confluence.

In contrast to the limited overexpression of TPO with
the membrane-associated form of the enzyme, overexpression of
the secreted form of hTPO by CHO-HTPO-M1 cells was much

15  greater. In these cells, most of the TPO is secreted into
the medium, with little remaining in the cells. TPO
expression increased markedly over baseline beginning at 333
nM MTX, with progressive increments up to the highest
concentration of used (10 uM). Slot-blot analysis of genomic

20  DNA from CHO-HTPO-M1 cells using either a dhfr or hTPO DNA
probe revealed similar amplification patterns parallel to
that of the pattern of TPO protein expression.

A comparison was made of the amount of TPO available
from the membrane-associated (CHO-HTPO-2B cells) and secreted

25  protein (CHO-HTPO-M1 cells) for immunological detection in an
ELISA. Three-day conditioned media from a single 100 mm dish
of confluent CHO-HTPO-M1 cells (10 uM MTX) yielded
significantly more TPO protein than did microsomes prepared
from a 100 mm confluent dish of CHO-HTPO-2B cells (100 nM

30  MTX). Both of these cell lines represented their highest
levels of TPO expression.

EXAMPLE XVI

The Role of Carbohydrate Moieties in Recognition of TPO by
Anti-TPO Antibodies in Hashimoto's Thyroiditis

5          Carbohydrate moieties on hTPO may contribute to the
epitopes recognized by anti-hTPO antibodies in Hashimoto's
thyroiditis.    This is because bacterial fusion proteins,
unlike proteins expressed in eukaryotic cells, are not
glycosylated.   Very little is known about the carbohydrate
10     moieties in hTPO. Human TPO (Ruf, J., et al., Acta Endocrinol.
Suppl. 281:49-56 (1987)) and the microsomal antigen (Kajita,
Y., et al., FEBS Lett. 187:334-338 (1985)) are bound to the
lectin concanavalin A. The latter is also partially bound to
lentil lectin (Kajita, Y., et al., FEBS Lett. 187:334-338
15     (1985)).     It is unknown whether the hTPO carbohydrate
structures are N-linked, O-linked, or both.  In the present
example, the nature of the carbohydrate components of hTPO was
examined, and whether or not hTPO carbohydrate plays a role in
the structure of naturally occurring epitopes in Hashimoto's
20     thyroiditis.

METHODS AND MATERIALS
       Cell   culture.   protein   radiolabeling   and   hTPO
immunoprecipitation:   Chinese hamster ovary (CHO) cells
25     stably expressing human hTPO (CHO-TPO 12g) (Kaufman, K.D., et
al., J. Clin. Invest. 84:394-403 (1989)) were cultured in 100
mm diameter dishes in F12 medium containing 10% fetal calf
serum, 100 U/ml penicillin, 40 μg/ml gentamicin and 2.5 μg/ml
amphotericin B.   For radiolabeling, subconfluent cells were
30     rinsed twice in phosphate-buffered saline without calcium and
magnesium (PBS-CMF), and were then incubated for 15-20 minutes
in methionine-free F12 medium (3 ml/dish) containing 10%
dialyzed fetal calf serum.   $^{35}$S-methionine (>1100 Ci/mmol;
Amersham, Arlington Heights, IL) was then added to the medium

(0.2 mCi/ml), and the incubation was continued for 2-4 hours
at 37°C. The medium was removed and the cells were rinsed
twice in PBS-CMF, scraped into ice-cold PBS-CMF, pelleted for
10 minutes at 1000 x g (4°C), washed once in 10 ml of the same
5    buffer, and the cell pellet resuspended (0.3 ml/dish of cells)
in homogenization buffer (50 mM Hepes, pH 7.5, 1% Triton X-
100, 0.1 mM phenylmethylsulfonyl fluoride, 2 mg/ml bacitracin,
0.25 mM TLCK (N-p-tosyl-1-lysine chloro-methyl ketone) and 0.1
mM leupeptin (Sigma Chemical Co., St. Louis, MO).    After
10   shaking for 1 hour at room temperature, the mixture was
centrifuged for 1 hour at 100,000 x g (4°C), and the
supernatant was diluted to 1 ml in immunoprecipitation buffer
(10 mM Na phosphate, pH 7.2, 1 M NaCl, 0.1% Na dodecylsulfate,
0.5% NP-40 and 2 mM EDTA).

15       The 1 ml of solubilized cellular proteins was pre-
adsorbed twice for 10 minutes at room temperature with 80 ul
of 10% IgG-Sorb (Staphylococcus A) (The Enzyme Center, Malden,
MA), followed by removal of the IgG-Sorb by centrifugation
for 3 minutes at 10,000 x g in a microfuge.   Hashimoto's
20   thyroiditis sera with high titers (ELISA readings >1.5 O.D.
units) of anti-hTPO antibodies were added to a final dilution
of 1:200.   Similar results were obtained with three separate
sera.   After incubation overnight at 4°C, 150 μl of IgG-Sorb
were added, and the tubes rotated end over end for 2-4 hours
25   at room temperature.    The IgG-Sorb was recovered by
centrifugation for 5 minutes at 10,000 x g, washed 5 times
with 1 ml of immunoprecipitation buffer, and then once with 10
mM Tris, pH 7.5, 2 mM EDTA and 0.5% Na dodecylsulfate.
Finally, the pellet was resuspended in Laemmli sample buffer
30   (31), with 50 mM dithiothreitol (DTT), boiled for 3 minutes,
and applied to 6% polyacrylamide gels.   Molecular weight
markers (Sigma; St. Louis, MO) were as follows:   205 kD
myosin; 116 kD β-galactosidase; 97 kD phosphorylase b; 66 kD

bovine serum albumin; 45 kD ovalbumin.  Autoradiography was
performed with Kodak XAR-5 film.

     Enzymatic deglycosylation of immunoprecipitated human
TPO:  Recombinant, radiolabeled hTPO, immunoprecipitated and
bound to IgG-Sorb, as described above, was recovered in
enzymatic digestion buffers rather than in Laemmli sample
buffer.  Enzymatic digestions (18 hours at 37°C) were as
follows:  endoglycanase F (Boehringer-Mannheim, West Germany,
30 U/ml; in 100 mM Na phosphate buffer, pH 6.0, 50 mM EDTA,
0.1% SDS, 1% beta-mercaptoethanol and 1% NP40); endoglycanase
H (Boehringer, 0.2 U/ml; in the same buffer as for endo F,
except that EDTA was omitted); O-glycanase (Boehringer, 2.5
U/ml; same buffer as for endo H); and neuraminidase (Sigma, 1
U/ml; in 100 mM Na acetate, pH 5.2, 5 mM EDTA and 1% $\beta$-
mercaptoethanol).  As a control, to monitor degradation of
the hTPO, each experiment included a sample incubated in
parallel without added enzyme.

     Lectin affinity chromatography:  Detergent extracts of
CHO-TPO cells (5-7 100 mm diameter dishes) were radiolabeled
with $^{35}$S-methionine (see above) and applied to 2 ml bed
volume columns of Concanavalin A (Con A), peanut agglutinin
(PNA), wheat germ agglutinin (WGA), Ricinus communis agglutin
1 (RCAl) and Ulex Europaeus (UEA-F) agarose-bound lectins
(all purchased from Vector Laboratories, Burlingame, CA).
For application to the columns, samples (0.3 ml) were diluted
to 10 ml in Buffer A (20 mM Tris HCl, pH 7.4, 150 mM NaCl,
0.1% Triton X-100), supplemented with the following for each
individual lectin:  WGA and RCAl- 1 mM EDTA; Con A - 1mM
$CaCl_2$, 1 mM $MnCl_2$; PNA - 1mM $CaCl_2$, 1 mM $MgCl_2$; UEA-F - 1mM
$CaCl_2$.  After application to the columns, the unbound
proteins were removed by washing with 50 ml of the foregoing
Buffer A's.  Specifically adsorbed proteins were eluted with
25 ml of the following (all at 300 mM):- WGA, N-
acetylglucosamine; PNA and RCAl, D-galactose; Con A, $\alpha$-

methyl-D-mannoside; and UEA-F, α-fucose. Fractions of 0.5 ml
were collected and counted for radioactivity in a liquid
scintillation counter. The two fractions containing the peak
of the eluted radioactivity were pooled (1 ml) and subjected
5    to immunoprecipitation with anti-hTPO antibodies in the sera
of patients with Hashimoto's thyroiditis, followed by
polyacrylamide electrophoresis and autoradiography (see
above).

10   RESULTS
        As described above, the derived amino acid sequence of
human TPO (Magnusson, R.P., et al., Mol. Endocrinol. 1:856-861
(1987); Kimura, S., et al., Proc. Natl. Acad. Sci. USA
84:5555-5559 (1987); Libert, F., et al., Nucl. Acids Res.  :
15   15:6735 (1987)) suggests that there are 5 potential
glycosylation sites in the extracellular domain of the enzyme.
This is based on the tri-peptide algorithm for glycosylation
sites of Asn-X-Ser/Thr (X refers to any amino acid; the third
position can be either Ser or Thr). Carbohydrate chains can be
20   linked to the Asn residue (N-linked), or to the Ser or Thr
residues (O-linked).
        To determine whether hTPO carbohydrate moieties were N-
linked, O-linked, or both, and also to obtain information
about the characteristics of the carbohydrate component(s),
25   hTPO was digested with a number of deglycosylating enzymes of
varying specificity. To prepare radiolabeled hTPO, proteins
in Chinese hamster ovary (CHO) cells expressing recombinant
hTPO were radiolabeled with $^{35}$S-methionine, followed by
immunoprecipitation with anti-hTPO antibodies present in the
30   serum of patients with Hashimoto's thyroiditis. As observed
previously on western blot analysis (Kaufman, K.D., et al., J.
Clin. Invest. 84:394-403 (1989)), recombinant hTPO was
present as a doublet of approximately 115 kD and 110 kD, with
the relative dominance of the 115 kD and the 110 kb bands

varying from experiment to experiment. Digestion with
endoglycosidase (endo) F, which removes both complex and
polymannose (Thotakura, N.R., et al., Meth. Enzymol. 138:350-
359 (1987)) N-linked glycans by cleaving the glycosidic
linkage between the two N-acetyl glucosamine (GlcNac) residues
in the chitobiose core, increased the electrophoretic mobility
of the hTPO doublet to approximately 110 kD and 105 kD. Endo
H, which acts similarly to endo F on polymannose but
differently from endo F on complex glycans, also converted the
mobility of hTPO to a 110 kD and 105 kD doublet. In contrast,
O-glycanase and neuraminidase, which remove O-linked glycans
and terminal neuraminic acid, respectively, did not alter the
mobility of radiolabeled hTPO. These data suggest that human
TPO contains only polymannose N-linked glycans.

        Lectin affinity chromatography (Merkle, R.K., et al.,
Meth. Enzymol. 138:232-259 (1987)) provided further support
for the polymannose nature of the hTPO carbohydrate moieties.
Thus, radiolabeled, recombinant hTPO was retained only on
concanavalin A-Sepharose, which binds with high affinity to N-
linked oligosaccharides in which at least two outer mannose
residues are either unsubstituted, or are substituted only at
position C-2 by another sugar. Bound hTPO could be eluted
with 300 mM α-methyl-D-mannoside. TPO did not bind to wheat
germ agglutinin (specificity for terminal and internal GlcNac
and terminal neuraminic acid), Ricinus communis agglutinin 1
(RCA1) (highest affinity for bi- and tri-antennary N-linked
oligosaccharides with terminal galactose residues), peanut
agglutinin (terminal Gal-β-1,3-GalNac) or Ulex europaeus
(terminal α-L-fucose).

        Having determined the type of carbohydrate present in
recombinant human TPO, the inventor investigated whether these
residues play a role in the disease-associated epitopes on
hTPO that are recognized by anti-hTPO antibodies in
Hashimoto's thyroiditis.

Radiolabeled recombinant hTPO was first partially
purified by concanavalin A-Sepharose affinity chromatography,
next digested with three different glycanases, and finally
subjected to immunoprecipitation with anti-hTPO antibody in
5    Hashimoto's thyroiditis serum.  Complete removal of the N-
linked carbohydrate chains distal to the chitobiose core with
endo F and endo H did not prevent antibody binding.  In view
of the complexity of these experiments, it is important to
note the completeness of N-glycanase treatment.  Thus, after
10   deglycosylation, all of the hTPO immunoprecipitated was as the
smaller (110 kD and 105 kD) doublet.  As a further control,
digestion with O-glycanase led to the immunoprecipitation of
an unaltered hTPO form (115 kD and 110 kD).

15   DISCUSSION
Previous studies have shown that the thyroid microsomal
antigen (Kajita, Y., _et al._, _FEBS Lett._ _187_:334-338 (1985))
and immunopurified, non-recombinant human TPO (Ruf, J., _et_
_al._, _Acta Endocrinol. Suppl._ _281_:49-56 (1987)) are bound by
20   concanavalin A.  However, the present inventor is not aware
of other data on the nature of the oligosaccharide (glycan)
moieties in human TPO.  By taking advantage of the expression
of recombinant human TPO in non-thyroidal eukaryotic cells as
described hereinabove, the present data provide new
25   information on this subject.
Thus, by both glycan enzymatic digestion and by
differential lectin affinity chromatography, the data
presented in this example provide strong evidence that all the
carbohydrate moieties on hTPO are linked to Asn residues (N-
30   linked) and not to Ser or Thr (O-linked).  Furthermore, the
selective deglycosylation with endo H (Thotakura, N.R., _et_
_al._, _Meth. Enzymol._ _138_:350-359 (1987)), as well as the
selective adsorption to concanavalin A (Merkle, R.K., _et al._,

Meth. Enzymol. 138:232-259 (1987)), suggests that these N-linked oligosaccharides are of the polymannose variety.

Most important from the perspective of the pathogenesis of Hashimoto's thyroiditis, the present data indicate that the oligosaccharides present in hTPO do not significantly influence the epitopes recognized by anti-hTPO antibodies in the sera of patients with autoimmune thyroid disease, primarily Hashimoto's thyroiditis.

An assumption inherent in the present example is that the glycan components of recombinant hTPO are structurally the same as those in hTPO present in human thyroid cells in vivo. While it cannot be excluded that Chinese hamster ovary cells may glycosylate the hTPO polypeptide chain in a manner different from human thyroid cells, it is likely that any such differences would be minor. Thus, unlike the polypeptide glycosylation pattern in yeast and bacteria, glycosylation in eukaryotic Chinese hamster ovary cells would be very similar, if not identical, to that in human thyroid cells. Further support for this assumption is that recombinant hTPO in Chinese hamster ovary cells is functionally active, at the same level present in human thyroid cells in monolayer culture (Kaufman, K.D., et al., J. Clin. Invest. 84:394-403 (1989)). In addition, virtually all sera from patients with Hashimoto's thyroiditis that contain anti-microsomal antibodies can recognize this form of recombinant human TPO on western blot analysis (Kaufman, K.D., et al., J. Clin. Invest. 84:394-403 (1989)) or by ELISA. Thus, by definition, the recombinant human TPO of the present invention contains the relevant, disease-associated epitopes on hTPO.

The present findings that removal of the carbohydrate moieties on human TPO does not affect the antigenicity of the molecule with respect to recognition by anti-hTPO antibodies in Hashimoto's thyroiditis serum are consistent with data

obtained with tunicamycin (see Example XI). However, the
present data are much stronger.

The present data suggest that oligosaccharide components
in hTPO are not part of the "natural" epitopes recognized by
5    anti-hTPO antibodies in the sera of patients with autoimmune
thyroiditis.    However,  it  remains  possible  that  the
glycosylated portion of the molecule could influence the
interaction of the antibody with its epitope(s), such as by
altering the affinity of this interaction.   Although not
10   intending to be bound by any particular theory, there is
increasing  recognition  that  the  majority  of  epitopes
recognized by both polyclonal and monoclonal antibodies are
discontinuous.  That is, by folding of the polypeptide chain,
the three-dimensional structure of a protein may bring into
15   apposition, as an epitope, widely separated, "discontinuous"
regions of the polypeptide chain.   This three-dimensional
configuration may be lacking in peptide fragments, or may be
altered by the β-galactosidase component of the bacterial
fusion protein.

20   The present data relate to the recognition of epitopes on
TPO by antibodies (B-cell epitopes).   These B-cell epitopes
are now recognized to be distinct from epitopes presented to
T-cells in a major histocompatibility antigen (MHC) restricted
manner (Livingstone, A.M., et al., Ann. Rev. Immunol. 5:477-
25   501 (1987)).  B-cell epitopes are likely to be important in
mediating damage by the immune system to the thyroid gland,
while T-cell epitopes are likely to be relatively more
important in the initiation of the autoimmune process.

## EXAMPLE XVII

Identification and Sequencing of β-Cell Region Capable of
Binding β-Cell Epitope on Thyroid Peroxidase

The sequences disclosed by the invention in, inter alia,
Examples XIII and XIV provide for a method of identifying the
interaction responsible for the β-cell recognition of thyroid
peroxidase.

In detail, using the sequences disclosed in Examples XIII
and XIV of the preferred embodiments, it is possible to
isolate the proteins which bind to these sequences. This is
accomplished using methods, well known in the art, of
purifying a protein which binds to a specific DNA sequence.
Preferably, a protein which binds to a specific DNA sequence
is purified using affinity chromatography. Specifically, the
nine amino acid sequence corresponding to residues 713-721 of
thyroid peroxidase is immobilized on an appropriate matrix,
such as Sepharose, and used as an affinity matrix to purify
the proteins which bind to the particular sequence (Arcangioli
B., et al., Eur. J. Biochem. 179:359-364 (1989)).

Preferably, the DNA binding protein is extracted from
human β cells. The protein extract, obtained from the β cell,
is applied to a column which contains the immobilized DNA
sequence of interest. Proteins which are not capable of
binding to the DNA sequence are washed off the column.
Proteins which bind to the DNA sequence are removed from the
column using a salt gradient. The proteins eluted from such a
column are enriched for the proteins which bind to the
specific DNA sequences immobilized on the matrix. The DNA
binding protein is further purified using procedures well
known in the art, such as ion exchange chromatography, high
performance liquid chromatography, size exclusion
chromatography, and the like.

During the purification of the DNA binding protein, the
protein is assayed, for example, using the well known gel

retardation assay (Garner, M.M. et al., Nucl. Acid Res.
9:3047 (1981); Fried, M. et al., Nucl. Acid Res. 9:6506
(1981)), or other well known methods.

Once the DNA binding protein is purified, a partial
amino acid sequence is obtained from the N-terminal of the
protein.  Alternatively, the protein is tryptically mapped
and the amino acid sequence at one of the fragments is
determined by methods known in the art.

The deduced amino acid sequence is used to generate an
oligonucleotide probe.  The encoding sequence can be based on
codons which are known to be more frequently used by the
organism.  Alternatively, the probe can consist of a mixture
of all the possible codon combination which could encode the
polypeptide.  Such methods are well known in the art.

A probe complementary to the amino acid sequence is used
to screen either a cDNA or genomic library for the genomic
sequences which encode the DNA binding protein.  Once the gene
encoding the DNA binding protein has been obtained, the
sequence of the DNA is determined according to well known
methods.  The gene can be used to obtain large amounts of the
protein from a recombinant host, or the sequence can be used
in mutational analysis to further define the functional
regions within the protein which interacts with the DNA.

Alternatively, proteins which bind to β-cell epitope
(residues 713-712) are isolated by identifying a clone
expressing the protein using well known techniques such as
Southwestern blotting (Sharp, Z.D. et al., Biochim Biophys
Acta, 1048:306-309 (1990); Gunther, C. V. et al., Genes Dev.
4:667-679 (1990); and Walker, M.D. et al., Nucleic Acids Res.
18:1159-1166 (1990)).

In a Southwestern blot, a labeled DNA sequence is used to
screen a cDNA expression library whose expressed proteins have
been immobilized on a filter via colony or plaque transfer.
The labeled DNA sequences bind to colonies or plaques which

express a protein capable of binding to the particular DNA
sequence.   Clones expressing a protein which bind to the
labeled DNA sequence are purified and the cDNA insert which
encodes the DNA binding protein is isolated and sequenced.
5      The isolated DNA can be used to express large amounts of the
protein for further purification and study, used in isolating
the genomic sequences corresponding to the cDNA, or used to
generate functional derivative of the binding protein.

        The present invention is thus directed to DNA binding
10     proteins which can bind to the β-cell epitope and to
functional derivatives thereof.

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## EXAMPLE XVIII

### Cloning of a Human Fab Fragment Specific for Human Thyroid Peroxidase

IgG autoantibodies to thyroid peroxidase (TPO) are characteristic of patients with Graves' and Hashimoto's diseases and are implicated in autoimmune thyroid destruction (reviewed in DeGroot, L.J., and Quintans, J., *Endocr.Rev.* VOL.10:537-562 (1989)). The availability of monoclonal human TPO autoantibodies of the same class and high affinity as those present in serum would contribute significantly towards an understanding of the pathogenesis of these common autoimmune diseases. Unfortunately, despite numerous attempts using EB virus infection and/or cell fusion, only one cell line secreting IgG class human autoantibody to TPO has been produced, and this human-mouse hybridoma was unstable (Fukuma, N., Sarsero, D., Furmaniak, J., Pegg, C.A.S., McLachlan, S.M., and Rees Smith, B., "B and T cell epitopes on thyroid peroxidase," (P. Carayon and J. Ruf, eds.), *Colloque INSERM/John Libbey Eurotext Ltd.* 195-201 (1990)).

Recently a technique has been described for cloning heavy and light chain gene fragments in a bacteriophage expression library (Huse, W.D., Sastry, L., Iverson, S.A., Kang, A.S., Alting-Mees, M., Burton, D.R., Benkovic, S.J., and Lerner, R.A. *Science* VOL.246:1275-1281 (1989)). In the present example the use of this approach to clone a human Fab fragment specific for human TPO is described.

### MATERIALS AND METHODS

#### Construction of combinatorial H and L gene libraries: A cDNA library from the thyroid gland of a patient with Graves' disease, as described in Example I, was used as a source of cDNA coding for

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thyroid autoantibodies. The presence of thyroid autoantibody mRNA in this gland was previously suspected for two reasons. First, infiltrating thyroid lymphocytes are a major source of thyroid autoantibodies (McLachlan, S.M., McGregor, A., Rees Smith, B., and Hall, R. *Lancet* VOL.i:162-163 (1979); Atherton, M.C., McLachlan, S.M., Pegg, C.A.S.., Dickinson, A., Baylis, P., Young, E.T., Proctor, S.J., and Rees Smith, B. *Immunology* VOL.55:271-279 (1985)). Second, proteins expressed by this library were recognized by antiserum to human IgG. A combinatorial library of heavy (H) chain fragments and kappa light chain genes was produced using oligonucleotides and vector in the Immunozap Cloning Kit (Stratacyte, La Jolla, CA). Bacteriophage DNA (Maniatis, T:, Fritsch, E.F., and Sambrook, J. Cold Spring Harbor Laboratory, N.Y. (1982)) prepared from the Graves' thyroid cDNA library was used as template in the polymerase chain reaction (Saiki, R.K., Gelfand, D.N., Stoffel, S., Scharf, S.J., Higuchi, R., Horn, G.T., Mullis, K.B., and Erlich, H.A. *Science* VOL.239:487-491 (1988)). Heavy chain gene sequences were amplified in separate reactions using 4 different forward oligonucleotide primers corresponding to the relatively conserved amino terminus of the molecule and a reverse primer to the CH1 domain-hinge junction of IgG1. Kappa light chain genes were amplified using primers complementary to the sequence coding for the signal peptide/kappa light (L) chain junction and the carboxyl terminus. The combinatorial library was constructed according to the protocol of Stratacyte.

Screening for TPO binding : The unamplified combinatorial library was screened in XL1-Blue cells by conventional techniques (Maniatis *et al.*, *supra*) using highly-purified, secreted recombinant human TPO, prepared as described above, labelled with $^{125}I$ by the iodogen method (Salacinski, P.R.P., McLean, C., Sykes, J.E.C., Clement-Jones, V.V., and Lowry, P.J. *Anal.Biochem.* VOL.117:136-146 (1981)) to a specific activity of 10 uCi/ug. A plaque expressing a Fab fragment that bound radiolabeled TPO was

identified by autoradiography and was cloned to homogeneity.  The
affinity of  this TPO antibody was measured in a confluent plaque
lysis assay (~100 plaques per spot) by the addition of  unlabeled
TPO  $(10^{-10}$ M - $10^{-6}$ M) to the $^{125}$I-labeled TPO.  Densitometry was
performed (Biorad 620 video-densitometer) on  duplicate  spots  at
each  TPO  concentration  and  expressed  as  arbitrary  OD (optical
density) units.

    <u>Nucleic acid sequencing of H and L genes coding for human TPO
specific Fab fragment</u> : The nucleotide sequence of the cDNA of the
TPO-positive clone (SP2) was determined (Sanger, F., Nicklen,  S.,
and  Coulson,  A.R.    Proc.  Natl.  Acad.  Sci.  USA <u>VOL.</u>74:5463-5467
(1977)) in both directions following its  recovery  in  Bluescript
using the helper phage R408 (Stratacyte).


**RESULTS**

     Screening ~2 x $10^5$  plaques  yielded one (SP2) that bound
radiolabeled TPO. After cloning to homogeneity, the affinity  for
TPO  of  the  Fab  fragment  expressed  by  SP2  was determined by
competition studies with unlabeled recombinant TPO and  was  found
to  be ~$10^{-9}$ M (Fig. 21). The specificity of this interaction was
evident by the inability of the Fab fragment to bind  radiolabeled
thyroglobulin, another major thyroid autoantigen.

     Comparison  of  the  nucleotide sequence of the IgG heavy (H)
chain (Fig. 22) and light (L) chain (Fig. 23) with known  germline
sequences   characterizes   this   TPO   binding   Fab   fragment.
Specifically, the VH gene belongs to the  VHI  family  with  91.2%
homology  to  the  1-1 germline gene (Pascual, V., and Capra, J.D.
Adv. Immunol. <u>VOL.</u>49:1-74 (1991)).  The D segment  contains  three
of  the  5  nucleotide motifs shared by $D_M$, $D_N$ and $D_{LR}$ (GGTAT) and
$D_{LR}$ (TACTA, GTATG) (Pascual <u>et al</u>., <u>supra</u>).  Because of  very  low
homology  with reported D region nucleotide  sequences,  it  is
difficult to assign the D region of  SP2  to  a  particular  gene
family.   The  J segment is a JH3 which appears to be truncated at

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its 5' end (P). The light chain is coded for by a VKI which is 89.6% homologous with the germline gene HUMIGKLVJ (GenBank accession number D90158). The light chain J segment is a JK2 (Kipps, T.J., Tomhave, E., Chen, P.P., and Fox, R.I. J.Immun. VOL.142:4261-4268 (1989)).

## DISCUSSION

The expression in bacteria of random combinations of heavy and light chain immunoglobulin cDNA genes has previously been used to generate human Fab fragments which bind tetanus toxoid using cDNA from individuals immunized with this antigen (Mullinax, R.L., Gross, E.A., Amberg, J.R., Hay, B.N., Hogrefe, H.H., Kubitz, M.M., Greener, A., Alting-Mees, M., Ardourel, D., Short, J.M., Sorge, J.A., and Shopes, B. Proc. Natl. Acad. Sci. USA VOL.87:8095-8099 (1990); Persson, M.A.A., Caothien, R.H., and Burton, D.R. Proc. Natl. Acad. Sci. USA VOL.88:2432-2436 (1991)). However, there are no previous reports on the production of disease-associated human autoantibodies using this system. In the present study, a human Fab fragment has been generated which binds a major thyroid autoantigen, TPO. The cDNA used for this purpose was transcribed from mRNA prepared from Graves' thyroid tissue which is enriched in B-lymphocytes capable of producing thyroid autoantibodies (McLachlan et al., supra; Atherton et al., supra).

Antibodies to TPO in patients with autoimmune thyroid disease are frequently of subclass IgG1 and/or IgG4, with kappa light chains predominating (Parkes, A.B., McLachlan, S.M., Bird, P., and Rees Smith, B. Clin. Exp. Immunol. VOL.57:239-243 (1984)). For this reason the initial approach undertaken was to construct and screen an IgG1-kappa combinatorial cDNA library. On the basis of available information, the VH and VL genes of SP2 appear to be moderately mutated forms of germline gene families VHI and VKI. The D region, which does not resemble any reported germline sequence, is likely to contribute further to recognition of TPO by

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SP2. It is not known whether this particular heavy and light chain combination reflects the _in vivo_ situation. However, the high affinity of SP2 is comparable to reported affinities of TPO autoantibodies present in patients with autoimmune thyroid disease (1.1 x $10^{-9}$ M - 9.4 x $10^{-8}$ M)(Beever, K., Bradbury, J., Phillips, D., McLachlan, S.M., Pegg, C., Goral, A., Overbeck, W., Feifel, G., and Rees Smith, B. _Clin. Chem._ VOL.35:1949-1954 (1989)). Therefore, the present example is belived to provide the first characterization at a molecular level of a human thyroid peroxidase antibody associated with autoimmune thyroid disease.

## EXAMPLE XIX

### Recognition by Recombinant Autoimmune Thyroid Disease-Derived Fab Fragments of a Dominant Conformational Epitope on Human Thyroid Peroxidase

Definition of the epitopes recognized by TPO autoantibodies is an important goal in understanding the pathogenesis of autoimmune thyroid disease. TPO autoantibodies appear to interact with different regions of the molecule. For example, some, but not all, TPO autoantibodies inhibit TPO enzymatic activity (Kohno, Y., Y. Hiyama, N. Shimojo, H. Niimi, H. Nakajima, and T. Hosoya, "Autoantibodies to thyroid peroxidase in patients with chronic thyroiditis: Effect of antibody binding on enzyme activities, Clin. Exp. Immunol. VOL.65:534-541 (1986)) and cross-react with myeloperoxidase or lactoperoxidase (Banga, J.P., N. Doble, R.W.S. Tomlinson, E. Odell, and A.M. McGregor, "Thyroid microsomal/ thyroid peroxidase autoantibodies show discrete patterns of cross-reactivity to myeloperoxidase lactoperoxidase and horseradish peroxidase," Immunology VOL.67:197-204, (1989)). At the molecular level, a linear epitope for serum TPO autoantibodies was defined in the preceeding example.

Human, disease-associated monoclonal TPO autoantibodies will be invaluable in defining TPO epitopes. However, as described above, such monoclonal antibodies are exceptionally difficult to generate by conventional techniques. The preceeding example describes the obtension of a human Fab fragment (designated "SP2" therein and also designated herein as "SP1.2") specific for TPO from B-cells infiltrating Graves' thyroid tissue. In the present example, the isolation of two additional TPO-specific Fab fragments (designated herein as "SP4" and "SP5," the same fragments also designated herein as "SP1.4" and "SP1.5," respectively) from a bacteriophage lambda library is described, as

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well as the expression and characterization of all three Fab
fragments. The data indicate that TPO autoantibodies represented
by these Fab fragments are present in all patients studied,
constitute a high proportion of serum TPO autoantibodies in
individual patients and interact with a conformational epitope on
TPO.


MATERIALS AND METHODS

Molecular cloning of TPO-binding Fab fragments:  Construction of
the IgG1/kappa Fab fragment combinatorial cDNA library in the
vector Immunozap (Stratacyte, La Jolla, CA) has been described
above.  The source of the mRNA was Graves' thyroid tissue, known
to contain IgG secreting cells.  The unamplified combinatorial
library (3 x 10^6 recombinants) was screened in XL1-Blue cells by
conventional techniques (Maniatis, T., E.F. Fritsch, and J.
Sambrook, "Molecular cloning. A laboratory manual," Cold Spring
Harbor Laboratory. Cold Spring Harbor Laboratory, N. Y., (1988))
using secreted recombinant human TPO as described in a preceding
example, affinity purified with mouse monoclonal anti-TPO, labeled
with $^{125}I$ to a specific activity of ~10uCi/ug protein by the
iodogen method (Salacinski, P.R.P., C. McLean, J.E.C. Sykes, V.V.
Clement-Jones, and P.J. Lowry, "Iodination of proteins,
glycoproteins, and peptides using a solid-phase oxidizing agent,
1,3,4,6-tetrachloro-3 alpha, 6 alpha-diphenyl Glycoluril
(Iodogen)," Anal. Biochem. VOL.117:136-146 (1981)).  TPO-binding
plaques were cloned to homogeneity and plasmids (Bluescript SK-)
were excised from the Immunozap bacteriophage using the helper
phage R408, according to the Stratacyte protocol. Nucleotide
sequences were determined in both directions (Sanger, F., S.
Nicklen, and A.R. Coulson, "DNA sequencing with chain terminating
inhibitors," Proc. Natl. Acad. Sci. USA VOL.74:5463-5467 (1977)).
    Preparation of soluble Fab fragments:  Plasmid-bearing XL1
Blue cells were incubated overnight at 37 C in LB medium

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containing 1% glucose. After dilution in LB medium without glucose, cultures were further incubated at 37 C until the optical density of the cells reached 0.5 (600 nm). Protein synthesis was induced with 1 mM isopropyl b-D-thiogalacto-pyranoside (Sigma Chemical Co., St. Louis, MO) for 1 h at 37 C. The cells were then pelleted, frozen at -20 C, resuspended in 0.02 volumes of 10 mM Tris pH 8.0 containing 2 ug/ml aprotinin, 1 ug/ml leupeptin, 1 ug/ml pepstatin, 0.1 mM phenylmethylsulfonyl fluoride (all from Sigma). The suspension was sonicated, membranes pelleted by centrifugation at 4000 x g and the Fab fragments affinity purified from the supernatant using a Protein G sepharose column (Pharmacia, Piscataway, NJ). The Fab proteins were ~99% pure as determined by SDS polyacrylamide gel electrophoresis (Laemmli, U.K., "Cleavage of structural proteins during the assembly of the head of bacteriophage T4," *Nature* VOL.227:680-685 (1970)).

Fab fragment binding to radiolabeled TPO: The interaction of the Fab fragments with ^{125}I-TPO was investigated as previously described for thyroglobulin monoclonal autoantibodies (Fukuma, N., S.M. McLachlan, V.B. Petersen, P. Kau, J. Bradbury, M. Devey, K. Bleasdale, P. Grabowski, and B. Rees Smith, "Human thyroglobulin autoantibodies of subclasses IgG2 and IgG4 bind to different epitopes on thyroglobulin," *Immunology* VOL.67:129-131 (1989)). Briefly, duplicate aliquots of SP1.2, SP1.4 or SP1.5, serially diluted in assay buffer (0.15 M NaCl containing 10 mM Tris-HCl pH7.5 and 0.5% bovine serum albumin), were incubated with ^{125}I-TPO (~ 25,000 cpm) and mouse monoclonal antibody to human kappa light chains (QE11, Recognition Sciences, Birmingham, U.K.) in a total volume of 200 ul. After 1 hour at room temperature, 100 ul donkey anti-mouse Sac-cel (IDS, Boldon, Tyne and Wear, U.K.) was added, and the incubation continued for 30 minutes. After vortexing, the mixture was centrifuged for 5 minutes at 1000 x g to sediment the immune complexes which were then counted to determine the percentage of radiolabeled TPO bound. The specificity of this

interaction was assessed by the addition of increasing amounts
($10^{-12}$ M - $10^{-8}$ M) of unlabeled recombinant human TPO, human
myeloperoxidase (Calbiochem, La Jolla, CA) or bovine
lactoperoxidase (Sigma). The affinities of the Fab fragments for
TPO were determined by Scatchard analysis (Scatchard, G., "The
attractions of proteins for small molecules and ions," *Ann. NY
Acad. Sci.* VOL.51:660-672 (1949)).

   Competition studies between Fab fragments and serum TPO
autoantibodies: Sera from 11 patients with autoimmune thyroid
disease were studied. All sera contained high levels of TPO
autoantibodies [detectable by ELISA (Schardt, C.W., S.M.
McLachlan, J. Matheson, and B. Rees Smith, "An enzyme-linked
immunoassay for thyroid microsomal antibodies, *J. Immunol. Methods*
VOL.55:155-168 (1982)) at dilutions of 1:1000 or greater].
Binding of [125] I-TPO by serum autoantibodies was measured by
precipitating the antigen-antibody complex with Protein A
(Pansorbin, Calbiochem)(Beever, K., J. Bradbury, D. Phillips, S.M.
McLachlan, C. Pegg, A. Goral, W. Overbeck, G. Feifel, and B. Rees
Smith, "Highly sensitive assays of autoantibodies to thyroglobulin
and to thyroid peroxidase," *Clin. Chem.*, VOL.35, 1949-1954,
(1989)) in the presence of increasing concentrations of Fab
fragments. The Fab fragment-TPO complex, lacking the CH2 domain
of the Fc region, is not precipitated by Pansorbin. Duplicate
aliquots of sera were incubated for 1 h at room temperature with
^{125}I-TPO, alone or with Fab fragments. Pansorbin (100 ul) was
added and the incubation continued for 30 min. After addition of
1 ml assay buffer (see above), the mixture was vortexed,
centrifuged for 30 min at 1000 x g (4 C), supernatants removed by
aspiration and TPO remaining in the pellets counted. In
preliminary experiments, serum dilutions needed to provide binding
values of ~ 20% in the absence of Fab fragments were determined.
These dilutions ranged from 1:600 - 1:2000. Non-specific ^{125}I-TPO
binding in the presence of control serum without TPO antibodies

was 2 - 5% of total cpm added. This value was subtracted from the values obtained with patients' sera in calculating the percentage inhibition by the SP Fab fragments.

Competition studies between Fab fragments and serum TPO antibodies of different IgG subclasses: An ELISA was used for this purpose, similar to that previously described for TPO autoantibodies measured using thyroid microsomal antigen (Parkes, A.B., S.M. McLachlan, P. Bird, and B. Rees Smith, "The distribution of microsomal and thyroglobulin antibody activity among the IgG subclasses," *Clin. Exp. Immunol.* VOL.57:239-243 (1984)), with some modifications. Conditioned medium (50ul per well) from CHO cells overexpressing a secreted form of TPO (described in a previous example), was used to coat ELISA plates (Immulon 4, Dynatech Laboratories, Chantilly, VA) by incubation overnight at 4 C with an equal volume of bicarbonate buffer (pH 9.3). Serum TPO autoantibodies of different IgG subclasses were detected with murine monoclonal antibodies (Jefferis, R., et al., "Evaluation of monoclonal antibodies having specificity for human IgG sub-classes: results of an IUIS/WHO collaborative study," *Immunol. Letters* VOL.10:223-252 (1985)) to human IgG1 and IgG4 (HP6012 and HP6011, respectively; Unipath Inc., Nepean, Ontario, Canada) and IgG2 (HP6014; Sigma). Serum TPO autoantibodies with lambda light chains were detected using murine monoclonal anti-human lambda (312H, Recognition Sciences). The signal was developed with affinity-purified anti-mouse IgG conjugated to horse radish peroxidase (Sigma) and o-phenylene diamine + $H_2O_2$ as substrate and optical densities (O.D.) read at 492 nm. None of these murine monoclonal antibodies bound to the Fab fragments. Competition studies were performed by incubating sera (appropriately diluted) with or without increasing concentrations of SP1.2 Fab fragment.

Screening of TPO cDNA fragment library: Construction of the library and the method used for screening has been described

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above. Reactivity of Fab fragment SP1.2 was assessed using the
murine anti-kappa antibody (QE11; Recognition Sciences) followed
by affinity-purified anti-mouse IgG horse-radish peroxidase
conjugate (Cappel, West Chester, PA). As a positive control, a
murine monoclonal antibody (#40.28), generated against denatured
TPO (Portmann, L., F.W. Fitch, W. Havran, N. Hamada, W.A.
Franklin, and L.J. DeGroot, "Characterization of the thyroid
microsomal antigen, and its relationship to thyroid peroxidase,
using monoclonal antibodies," *J. Clin. Invest.* VOL.81:1217-1224
(1988)) was used which recognizes a linear epitope expressed in
this library.

   Interaction between Fab fragments and denatured TPO:   TPO.in
conditioned culture medium (described above) was reduced and
alkylated using dithiothreitol and iodoacetamide (Nakajima, Y.,
R.D. Howells, C. Pegg, E. Davies Jones, and B. Rees Smith,
"Structure activity analysis of microsomal antigen/thyroid
peroxidase," *Molec. Cell. Endocrinol.*, VOL.53, 15-23, (1987)).
ELISA plates were coated with native or denatured TPO, as
described above. Binding of SP1.2, SP1.4 and SP1.5 ($10^{-9}$ M) was
detected, as described above for serum TPO lambda autoantibodies,
using monoclonal anti-kappa (QE11). Mouse monoclonal antibody
(#40.28, diluted 1:100) and control ascites (NS-1, diluted 1:50;
Cappel) were included as positive and negative controls
respectively.

RESULTS
Nucleotide and derived amino-acid sequences of TPO-binding Fab
fragments: Further screening of the Fab fragment combinatorial
cDNA library for radiolabeled TPO binding yielded two additional
Fab fragments (SP1.4 and SP1.5). The heavy chains of SP1.4 and
SP1.5 were identical to that of previously isolated SP1.2. The
light chains of SP1.4 and SP1.5, although closely related to that
of SP1.2, differed in their nucleotide (Fig. 24 and 26) and

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derived amino acid (Fig. 25 and 26) sequences. All 3 light chains
are coded for by VK1 and JK2, the closest germ-line genes being
HUMIGKLV (GenBank accession #D90158) and KV312 (Kipps, T.J., E.
Tomhave, P.P. Chen, and R.I. Fox, "Molecular characterization of a
major autoanitbody-associated cross-reactive idiotype in Sjogren's
Syndrome," J. Immun. VOL.142:4261-4268 (1989)), respectively.

Regarding the VK region, the SP1.4 and SP1.5 amino acid
sequences are more homologous (90%) to HUMIGKLV than SP1.2 (83%).
Only the kappa chain of SP1.2 has a potential glycosylation site,
N-X-S, in the CDR1 (Fig. 25). Unlike SP1.2, in which the J
segment is identical to the germline gene KV312, SP1.4 and SP1.5
(which are identical to each other) have 4 nucleotide
substitutions, one of which results in a L to V change (Fig. 26).

Specificity and affinity of TPO-binding Fab fragments:
Binding of the SP1.2 Fab fragment to $^{125}$I-TPO was readily
displaced by unlabeled TPO but not by myeloperoxidase or
lactoperoxidase (Fig. 27A). SP1.4 and SP1.5 displayed the same
specificity for TPO. As determined by Scatchard analysis, the
affinities for TPO of all 3 Fab fragments were remarkably high
(Fig. 27B). SP1.2 and SP1.5 displayed similar affinities (Kd $^{-}$6-8
x $10^{-11}$M). The affinity of SP1.4 was slightly lower ($^{-}$2 x $10^{-10}$
M).

Competition studies between Fab fragments and serum TPO
autoantibodies: We determined the abilities of SP1.2, SP1.4 or
SP1.5 to compete with TPO autoantibodies in patients' sera for
binding to $^{125}$I-TPO. Increasing amounts of SP1.2 ($10^{-10}$ to $10^{-7}$
M) progressively inhibited the binding to TPO by all of 11 sera
from patients with autoimmune thyroid disease that were tested
(Fig. 28). In the individual 11 sera, maximum inhibition ranged
from 36% to 72%. The results obtained for 5 of these sera were
similar with all 3 Fab fragments: 55 $\pm$ 2% (mean % inhibition $\pm$
S.E.M.) for SP1.2, 49 $\pm$ 8% for SP1.5 and 43 $\pm$ 6% for SP1.4.
Because SP1.2, SP1.4 and SP1.5 have identical heavy chains and

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similar light chains belonging to the same VK gene family, it is
reasonable to assume that they bind to the same, or closely
related, epitope. This assumption is supported by studies in mice
demonstrating that high affinity binding to a specific epitope is
usually achieved by re- combination of the same heavy chain with
light chains of the same family (Smith-Gill, S.J., P.A. Hamel,
T.B. Lovoie, and K.J. Dorrington, "Contributions of immunoglobulin
heavy and light chains to antibody specificity for lysozyme and
two haptens," *J. Immun.* VOL.139:4135-4144 (1987)).

Competition studies between Fab fragments and serum TPO
autoantibodies of different subclasses and lambda light chain:
Sera from individual patients contain different proportions of TPO
antibodies of subclasses IgG1 and IgG4 and, in some patients, IgG2
(Parkes et al, Weetman, A.P., C.M. Black, S.B. Cohen, R.
Tomlinson, J.P. Banga, and C.B. Reimer, "Affinity purification of
IgG subclasses and the distribution of thyroid auto-antibody
reactivity in Hashimoto's thyroiditis," *Scand. J. Immunol.*
VOL.30:73-82 (1991)). Therefore, the effect of SP1.2 (as a
representative Fab) on the inhibition of TPO binding to serum TPO
autoantibodies of different subclasses was investigated. Data
illustrating 3 different inhibition patterns among the 9 sera
analyzed are shown (Fig. 28). In one patient (Fig. 28A), IgG4 TPO
antibody was inhibited to a greater extent (77%) than IgG1 TPO
antibody (14%). In contrast, in another patient (Fig. 28B), IgG1
TPO antibody binding was inhibited to a greater extent (62%) than
IgG4 TPO antibody (13%). Data on a third patient are shown (Fig.
28C) because this serum was one of three with detectable IgG2 TPO
antibodies. A small degree of inhibition of IgG2 was observed
(29%). The predominant inhibition for this patient was in the
IgG1 subclass.

In reported previously (Parkes et al., supra), the serum TPO
autoantibodies studied were predominantly of kappa light chain
type. Because SP1.2 is a kappa Fab fragment, it was not possible

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to assess inhibition of serum kappa autoantibody binding to TPO. However, it was possible to investigate whether or not SP1.2 could compete with serum lambda TPO autoantibody binding to TPO. The extent of this inhibition was small, ranging from 12-18% in 4 sera.

Studies on the nature of the epitope recognized by TPO-specific Fab fragments: SP1.2 failed to recognize any plaques when used to screen a library of TPO cDNA fragments (66-166 amino acids in length) expressed in bacteria, as described above. In contrast, the positive control, murine monoclonal antibody #40.28, reacted with 5/~2000 plaques. These data suggested that the epitope recognized by SP1.2 is non-linear.

Confirmation that the epitope recognized by SP1.2 (and also by SP1.4 and SP1.5) is conformational was obtained in ELISA studies using native recombinant TPO and TPO denatured by reduction and alkylation. The three Fab fragments bound less well to denatured than to native TPO (Fig. 29). In contrast, binding of mouse monoclonal #40.28 was higher to denatured than to intact TPO.

DISCUSSION

The TPO-specific Fab fragments SP1.2, SP1.4 and SP1.5 all have the heavy chain variable region sequence described in a previous example dor SP1.2. The VH region is a member of the VHI family (91.2% homologous to the 1-1 germ-line gene) and the J segment is a JH3 with a truncation at its 5' end (Pascual, V., and J.D. Capra, "Human immunoglobulin heavy chain variable region genes: Organization, polymorphism and expression," *Advances in Immunology* VOL.49:1-74 (1991)). Because of its contribution to CDR3, the D region of these high affinity antibodies, which is not a member of any families reported, is of interest. The SP D region does not appear to result from homologous recombination (Sanz, I., "Multiple mechanisms participate in the generation of

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diversity of human H chain CDR3 regions," *The Journal of Immunology*  VOL.14786:1720-1729 (1991)) of known D segments. However, there is modest homology with a portion of the very long D segment of a cytomegalovirus antibody (Newkirk, M.M., H. Gram, G.F. Heinrich, L. Ostberg, J.D. Capra, and R.L. Wasserman, "Complete protein sequences of the variable regions of the cloned heavy and light chains of a human anti-cytomegalovirus antibody reveal a striking similarity to human monoclonal rheumatoid factors of the Wa idiotypic family," *J. Clin. Invest.* VOL.81:1511-1588 (1988)) and SP D shares the motif GGTAT in families $D_M$, $D_N$ and $D_{LR}$ and the motifs TACTA and GTATG in $D_{LR}$ (Pascual et al., *supra*).

The light chains of SP1.2, SP1.4 and SP1.5, though not identical, are closely related, moderately mutated members of the VKI and JK2 families. There is no relationship between the extent of mutation from the germline gene and their affinities for TPO. Overall, in accordance with previous data (Kabat, E.A., and T.T. Wu., "Identical V region amino acid sequences and segments of sequences in antibodies of different specificities," *J. Immun.* VOL.147:1709-1719 (1991)), the sequence information for both SP heavy and light chains suggests that one chain, in this case the heavy chain, is primarily responsible for SP specificity.

The SP Fab fragments were isolated from a library containing random combinations of heavy and light chain gene fragments. Therefore, it is not possible to determine whether or not the heavy and light chain combinations of the SP Fab fragments were present *in vivo* in the patient whose thyroid infiltrating B-cells were used to construct the combinatorial library. Nevertheless, the Fab fragments, particularly SP1.5 and SP1.2, have very high affinities for TPO, comparable to the highest affinity TPO autoantibodies reported for serum (Beever et al., *supra*). For this reason, as well as because of their derivation from a patient with autoimmune thyroid disease, the SP Fab fragments are suitable

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for investigating the interaction between TPO and serum TPO
autoantibodies.

The ability of the SP Fab fragments to inhibit the binding to
TPO of serum autoantibodies demonstrates identity or overlap
between the Fab epitope and an epitope recognized by serum
autoantibodies. Because this inhibition was observed for all 11
patients' sera examined, the epitope is very common in the TPO
autoantibody repertoire. The present data indicate that the SP
epitope is recognized by 36-72% of the TPO autoantibodies present
in the sera of individual patients. The present data also
indicate that patients' autoantibodies against the SP epitope are
not restricted to a single IgG subclass. Thus, in some patients
the SP epitope is predominantly IgG1, in others IgG4 and in a few
IgG2. In terms of light chain type, the SP epitope is recognized
poorly by TPO autoantibodies with lambda light chains. The SP
epitope-recognizing antibodies in the patient population are,
therefore, likely to contain kappa light chains, like the SP Fab
fragments themselves.

Techniques previously available to study TPO epitopes, such
as the use of synthetic peptides, proteolytic fragments and
recombinant TPO fragments generated by cDNA libraries, are only
capable of identifying linear epitopes. The present example
provides surprising and significant evidence that the SP epitope,
recognized by very common and abundant TPO autoantibodies, is not
linear but is conformational. Crystallization of the complex
between recombinant TPO-specific Fab fragments and recombinant TPO
will ultimately permit the definition of the amino acids involved
in the highly conformational epitopes on TPO.

## EXAMPLE XX

### Lack of Promiscuity in Autoantigen-Specific H and L Chain Combinations as revealed by Human H and L Chain "Roulette"

A hallmark of autoimmune thyroid destruction in man is the presence in serum of high affinity IgG class autoantibodies to thyroid peroxidase (TPO), the primary enzyme involved in thyroid hormone synthesis (reviewed in McLachlan, S.M., and B. Rapoport, "The molecular biology of thyroid peroxidase: cloning, expression and role as autoantigen in autoimmune thyroid disease," *Endocr. Rev.* VOL. 13:192 (1992), incorporated herein by reference as though set forth in full). As described above, TPO is a glycoprotein of ¯ 107 kD expressed on the surface of thyroid cells. Human autoantibodies to TPO are not monoclonal as evident by the contribution of different IgG subclasses and light chain types in the same patient (Parkes, A.B., S.M. McLachlan, P. Bird, and B. Rees Smith, "The distribution of microsomal antibody activity among the IgG subclasses," *Clin. Exp. Immunol.*, VOL.57:239 (1984); Weetman, A.P., C.M. Black, S.B. Cohen, R. Tomlinson, J.P. Banga, and C.B. Reimer, "Affinity purification of IgG subclasses and the distribution of thyroid auto-antibody reactivity in Hashimoto's thyroiditis," *Scand. J. Immunol.* VOL.30: 73 (1989)).

The preceding examples descibe the cloning of three human IgG1/kappa autoantibodies (SP1.2, SP1.4 and SP1.5; also designated SP2, 4 and 5, respectively) which bind TPO specifically and with high affinity. These autoantibodies were obtained by expressing random combinations of H and L chain Ig genes as antigen binding fragments, ("F(ab)s"), in a bacteriophage lambda library, the cDNA of which library was prepared from B cells infiltrating the thyroid gland in a patient with autoimmune thyroid disease. The three TPO autoantibody F(ab)s share the identical Ig H chain.

The L chains are not the same, but are derived from the same V kappa germline gene (HUMIGKLVJ) as described herein.

Because of the random nature of the H and L chain combinations in this cDNA library, the question arose as to whether or not the SP F(ab) autoantibody H chain (or L chain) could combine with a variety of other L chains (or H chains) in the parent library and still bind to TPO.

Recently it has been reported that there is notable promiscuity in the H and L chain combinations of murine F(ab)s which bind the hapten NPN (Kang, A.S., T.M. Jones, and D.R. Burton, "Antibody redesign by chain shuffling from random combinatorial immunoglobulin libraries," Proc. Natl. Acad. Sci. USA VOL.88:11120 (1991)). In contrast to this promiscuity, the "roulette" experiments described in the present example demonstrate more restriction in the H and L chain combinations for high affinity binding to a human autoantigen, TPO.

MATERIALS AND METHODS

Library construction and screening: The plasmid of clone SP1.2 was digested with Xho I and Spe I to release the H chain cDNA insert. Similarly, the L chain cDNA fragment was obtained by Xba I and Sac I digestion. The inserts were gel-purified and ligated into Immunozap H and L arms (Stratacyte, La Jolla, CA), respectively, to generate a clonal SP1.2 H and a clonal SP1.2 L chain library, respectively. These libraries were amplified and the DNA extracted as described above. The DNA from the SP1.2 heavy chain library was digested with HindIII followed by EcoRl and ligated with the DNA prepared from the original L chain library described above. Similarly the DNA from the SP1.2 light chain library was digested with MluI followed by EcoRl and ligated with the DNA from the original H chain library described above. The original H and L libraries contained >80% inserts of the correct size. The combinatorial libraries were screened in

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XL1-Blue cells by conventional techniques (Maniatis, T., E.F.
Fritsch, and J. Sambrook, "Molecular cloning: A laboratory
Manual," Cold Spring Harbor Laboratory. *Cold Spring Harbor
Laboratory, N.Y.* (1982)) using secreted recombinant human TPO as
dscribed above, labeled with $^{125}$I to a specific activity of 10-20
uCi/ug protein by the iodogen method (Salacinski, P.R.P., C.
McLean, J.E.C. Sykes, V.V. Clement-Jones, and P.J. Lowry,
"Iodination of proteins, glycoproteins, and peptides using a
solid-phase oxidizing agent, 1,3,4,6-tetrachloro-3 alpha, 6 alpha-
diphenyl Glycoluril (Iodogen)," *Anal. Biochem.* VOL.117:136
(1981)). TPO-binding plaques were cloned to homogeneity and
plasmids were excised from the Immunozap bacteriophage using the
helper phage R408, according to the Stratacyte protocol.
Nucleotide sequencing of the cDNA inserts was performed by the
dideoxynucleotide chain termination method (Sanger, F., S.
Nicklen, and A.R. Coulson, "DNA sequencing with chain terminating
inhibitors," *Proc. Natl. Acad. Sci. USA* VOL.74:5463 (1977)).

F(ab) expression: F(ab)s were expressed as soluble proteins
in XL1-Blue cells, as described above. In brief, protein
synthesis was induced with 1 mM isopropyl-thio-galacto-pyranoside
(Sigma Chemical Co., St. Louis, MO) for 1 h at 37 C. The cells
were then pelleted, frozen at -20 C, resuspended in 0.02 volumes
of 10 mM Tris pH 8.0 containing 2 ug/ml aprotinin, 1 ug/ml
leupeptin, 1 ug/ml pepstatin, 0.1 mM phenylmethylsulfonyl fluoride
(all from Sigma). The suspension was sonicated, membranes
pelleted by centrifugation at 4000 x g and the F(ab)s were
affinity purified from the supernatant using a Protein G sepharose
column (Pharmacia, Piscataway, NJ).

F(ab) binding of $^{125}$I-TPO: As previously described, F(ab)s
diluted in assay buffer (0.15 M NaCl containing 10 mM Tris-HCl
pH7.5 and 0.5% bovine serum albumin) were incubated with $^{125}$I-TPO
(~ 25,000 cpm) and mouse monoclonal antibody to human kappa light
chains (QE11, Recognition Sciences, Birmingham, U.K.) in a total

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volume of 200 ul. After 1 hour at room temperature, 100 ul donkey
anti-mouse Sac-cel (IDS, Boldon, Tyne and Wear, U.K.) was added,
and the incubation continued for 30 minutes. After addition of 1
ml assay buffer and vortexing, the mixture was centrifuged for 5
minutes at 1000 x g to sediment the immune complexes which were
then counted to determine the % radiolabeled TPO bound. The
affinities of the F(ab)s for TPO were determined by Scatchard
analysis (Scatchard, G, "The attractions of proteins for small
molecules and ions," Ann. NY Acad. Sci. VOL.51:660 (1949)) from
values obtained in the presence of increasing concentrations of
unlabeled TPO. The data presented are the mean $\pm$ SEM of
triplicate determinations.

Competition between F(ab)s for binding to TPO: One F(ab) was
immobilized by incubation (total volume of 200 ul) with murine mAb
anti-human kappa (QE11) for 1 h at room temperature. After
incubation with 100 ul of Sac-cel (30 min at room temperature),
the complexes were diluted in assay buffer (see above) and
centrifuged at 1000 x g (5 min at 4 C). The pellets were
resuspended in normal human serum diluted 1:30 in assay buffer to
saturate remaining anti-kappa binding sites. In a separate set of
tubes, increasing concentrations of "free" F(ab) were preincubated
with ^{125}I-TPO for 1 h at room temperature. Aliquots (100 ul) were
then incubated for 30 min with the immobilized F(ab) pellets,
washed with assay buffer and radioactivity bound to the Sac-cel
was counted. Non-specific binding (~2% of total counts added) was
subtracted to provide values for specific binding to TPO.

RESULTS

Frequencies of ^{125}I-TPO binding clones. Combining the L chain of
SP1.2 with the SP parent H chain library yielded a combinatorial
library of ~$10^7$ pfu (SP1.2 L x H library). Similarly, the
combination of the SP1.2 H chain with the SP parent L chain
library resulted in a library of ~$10^7$ pfu (SP1.2 H x L). As

expected, this "roulette" with either the SP1.2 L or H chain led
to a greater frequency of TPO-binding clones than was detected in
the original screening of the parent library (Table V). Further,
the frequency of TPO-binding was 10-fold greater in the SP1.2 H x
L library than in the SP1.2 L x H library.

   TPO  binding  clones  from  the  SP1.2  L  x  H  library.
Determination of the nucleotide sequences of the 6 new TPO-binding
clones isolated from the SP1.2 L x H library revealed that the VDJ
regions of 5 (SP1.7-11) were almost identical to those of the
original SP1.2. The VH of SP1.2 appears to be derived from the
VH1 family germline gene 1-1 (Pascual, V., and J.D. Capra, "Human
immunoglobulin heavy chain variable region genes: Organization,
polymorphism and expression," Advances in Immunology VOL.49:1
(1991)). In the VH region, 2 clones (SP1.7 and SP1.8) differed
from the original SP1.2 H chain by only one amino acid (Pro and
Leu for Ala 24, respectively). The D regions of SP1.7-11 are
identical and differ from the SP1.2 D region by a single silent
base substitution. The J regions of SP1.7-11 are of the JH6
family (Pascual et al., supra). Reexamination of the SP1.2 DJ
regions indicates that this clone, too, is a JH6 (rather than a
JH3) combined with a very short D region.

   One of the 6 new clones (SP4.6) from the SP1.2 L x H library
differed more substantially from SP1.2 as well as from SP1.7-11.
SP4.6 is a member of the VH1 family. However, it is more closely
related to the germline gene hv1L1 (Olee, T., E.W. Lu, D-F. Huang,
R.W. Soto-Gil, M. Deftos, F. Kozin, D.A. Carson, and P.P. Chen,
"Genetic analysis of self-associating immunoglobulin G rheumatoid
factors from two rheumatoid synovia implicates an antigen-driven
response," J. Exp. Med. VOL.1750:8310 (1992)) (88% homology) (Fig.
31A and B) than to 1-1. The D region does not resemble any
published D region sequence (Fig. 31 C). The SP4.6 J region is a
JH4 truncated at its 5' end. The most surprising finding was that
the hinge region sequence indicates that SP4.6 is an IgG4, in

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contrast to SP1.2 and SP1.7-11 which are IgG1. The C region
primer used for PCR in constructing the SP H chain library ("CH1";
Stratacyte, San Diego CA) is described as an IgG1 primer with the
capacity to cross-prime with other IgG subclasses.

TPO binding clones from the SP1.2 H x L library. The
nucleotide sequences of the L chains were determined in 11
TPO-binding clones in the SP1.2 H x L library. At the amino acid
level, the V kappa regions of 9 clones were very similar to, and 2
clones (SP1.17 and SP1.19) were identical to, the original SP1.2 L
chain (Fig. 32A). Four of the 9 clones (SP1.14, -15, -21 and -22)
have identical V kappa regions. All 11 new L chain clones appear
to be derived from the V kappa germline gene HUMIGKLVJ (GenBank
accession number D90158), as described above for SP1.2. At the
amino acid level, all 11 are more homologous with HUMIGKLVJ (83%
for SP1.18 to 92% for SP1.20) than is SP1.2 (81%). The J kappa
regions of 10 of the 11 new clones belong to JK1 or JK2 (Fig.
32B). The J kappa region of SP1.21 has not been assigned.
SP1.14, SP1.15 and SP1.22 have identical V kappa and J kappa
sequences. Because clones SP1.17 and SP1.19 have JK2 regions
(like SP1.2) these clones are, overall, identical to SP1.2.

Affinities for TPO of selected F(ab)s. The proteins for two
of the newly isolated F(ab) clones, SP4.6 and SP1.20, were
expressed and purified. SP4.6 was selected in view of its
distinctive H chain and SP1.20 because its L chain was the least
mutated from the putative germline gene. Purified SP1.2 is
described above. The affinities for TPO of the SP4.6 and SP1.20
F(ab)s, calculated by Scatchard analysis, are very similar to each
other (kD 1.0 $\pm$ 0.2 x $10^{-10}$ M and 1.4 $\pm$ 0.3 x $10^{-10}$ M,
respectively) and to SP1.2 (0.8 $\pm$ 0.1 x $10^{-10}$ M)(means $\pm$ SEM).

Binding domains on TPO for SP F(ab)s. To determine whether
or not there was overlap in the domains on TPO recognized by
SP4.6, SP1.20 and SP1.2, studies with an immobilized SP1.2 F(ab)
were performed. As expected, pre-incubation of $^{125}I$- TPO with

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increasing concentrations of free SP1.2 inhibited the subsequent binding of the antigen to the immobilized SP1.2 (Fig. 33). The SP4.6 and SP1.20 F(ab)s were equally effective, indicating that the binding domains of all 3 F(ab)s overlapped. No competition was observed with another anti-TPO F(ab) cloned from another patient.

## DISCUSSION

In the present example, a single H or L chain already known to confer high affinity ($\sim 10^{-10}$ M), specific binding for TPO was chosen. This H (or L chain) was used to search for other H (or H) chains that could form a F(ab) capable of binding TPO. That is, it was undertaken to "spin the wheel" of the H and L chain repertoire of activated B cells infiltrating the patient's thyroid gland. As expected, the frequency of TPO-binding clones in the libraries generated by this biased recombination was higher than in the original random combinatorial library. However, the frequency was surprisingly lower (Table V) than would be expected if promiscuous binding to a variety of H or L chain was compatible with specific antigen binding.

The antibody repertoire in thyroid tissue B cells of patients with autoimmune thyroid disease is relevant to this discussion. This tissue is enriched, compared with draining lymph nodes and peripheral blood, in B cells actively secreting autoantibodies to the three major thyroid autoantigens, TPO, thyroglobulin and the thyrotropin receptor (McLachlan, Rapoport et al., supra; McLachlan, S.M., A. McGregor, B. Rees Smith, and R. Hall, "Thyroid-autoantibody synthesis by Hashimoto thyroid lymphocytes," Lancet VOL.i:162 (1979)).

This bias makes even more remarkable the paucity of H and L chains from this patient capable of combining with the pre-selected L or H chain to form a functional TPO binding site. For example, frequencies of 1 in 50 for a functional H chain have

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been reported in libraries of a mouse immunized with influenza hemagglutinin or in a human immunized with tetanus toxoid (Kang et al., supra).

Nucleotide sequence analysis of the H and L chains of the new TPO binding clones reveals even more restriction. Thus, using the SP1.2 H chain, all 11 new clones utilized L chains from the same VK1 family germline gene. This germline gene was also used by the three other TPO F(ab)s described above. Amino acid substitutions predominate in the CDR regions. Overall, taking into account the V and J kappa regions of all 14 L chains, only 10 are distinct because several were identical to the SP1.2 L chain, or to each other.

Additional evidence for restriction was the very limited variety of H chains "captured" by the SP1.2 L chain in generating new TPO-binding clones. Five of the 6 clones were very closely related to the SP1.2 H chain, which is also shared by the previously identified SP1.4 and SP1.5 clones. However, in contrast to the L chains, one totally different H chain was isolated in SP4.6. This H chain differed from all others in the following respects: i) it is apparently derived from a different VH germline gene, although of the same VH1 family; ii) the D region is totally distinct; iii) it uses a different J region; and iv) it belongs to the IgG 4 subclass. This heavy chain is clearly more rare than the SP1.2 and related H chains, possibly because the oligonucleotide primer used to obtain the H chain library is suboptimal for IgG4. It is likely that this H chain was only found because of the bias introduced by the roulette approach.

In addition to providing data on the frequency and characterization of TPO-binding H and L chain combinations, the present example illustrates important features with respect to antibody affinity and binding domains. These TPO autoantibodies have affinities for antigen several orders of magnitude above those reported for naturally occuring, polyreactive autoantibodies

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(Kd $10^{-3}$ - $10^{-8}$ M) (Nakamura, M., S.E. Burastero, Y. Ueki, J.W. Larrick, A.L. Notkins, and P. Casali, "Probing the normal and autoimmune B cell repertoire with Epstein-Barr virus: frequency of B cells producing monoreactive high affinity autoantibodies in patients with Hashimoto's disease and systemic lupus erythematosus," J. Immun. VOL.141:4165 (1988)).

It is of interest that the affinity of SP1.20 is high (similar to SP1.2) even though its V kappa chain is less mutated than that of SP1.2 (92 % and 81% homology to the germline gene, respectively). Assuming that both genes are, indeed, derived from HUMIGKLVJ, it is possible that affinity maturation of the light chain does not play a critical role in determining the affinity of the F(ab) for TPO.

A most surprising and unexpected finding in the present example concerns the TPO binding domains of the SP F(ab)s. Thus, in accordance with the data of Radic et al. (Radic, M.Z., M.A. Mascelli, J. Erikson, H. Shan, and M. Weigert, "Ig H and L chain contributions to autoimmune specificities," J. Immun. VOL.146:176 (1991)) concerning murine autoantibodies to DNA, it was anticipated that both the SP1.2 and SP1.20 F(ab)s, which have the same H chain and have closely related L chains, would interact with overlapping epitopes and bind to the same domain on TPO. However, a similar domain on TPO was recognized by both SP4.6 and SP1.2 despite the fact that their V, D and J regions are quite different. This finding contrasts with the data of Martin et al. (Martin, T., S.F. Duffy, D.A. Carson, and T.A. Kipps, "Evidence for somatic selection of natural autoantibodies," J. Exp. Med. VOL.175, 983 (1992)), who found that polyspecific autoantibody activity could only be generated with a specific D region.

The results obtained in the present example also were unexpected in view of the extensive analysis of V region H and L chain combinations by Kabat and Wu (Kabat, E.A., and T.T. Wu, "Identical V region amino acid sequences and segments of sequences

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in antibodies of different specificities," J. Immun. VOL.147, 1709
(1991)), which suggested VH dominance in defining antibody
specificity in many instances. However, the present findings
raise the possibility that the L chain is critical in defining
epitope specificity, even in the presence of completely different
D regions and non-identical VH regions.

Table V: Frequencies of $^{125}$I-TPO binding clones in "roulette" of SP1.2 H and L chains.

| F(ab) Combinatorial Library | Frequency | Plaques Screened |
|---|---|---|
| SP H Chain x SP L Chain | 1:60,000 | 180,000 |
| SP1.2 L Chain x SP H Chain | 1:5,000 | 30,000 |
| SP1.2 H Chain x SP L Chain | 1:500 | 15,000 |

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## EXAMPLE XXI

**Human Organ-Specific Autoimmune Disease: Molecular Cloning of an Autoantibody Repertoire for a Major Autoantigen Establishes an Immunodominant Region**

The most common organ-specific autoimmune disease in man involves the thyroid. Hashimoto's thyroiditis and Graves' disease, in their pure forms, represent two ends of a clinical spectrum ranging from glandular hypofunction to hyperfunction. In both diseases there is a breakdown in tolerance to a number of thyroid-specific autoantigens and the generation of a marked, high-affinity IgG antibody response. Thyrotropin receptor autoantibodies occur predominantly in Graves' disease and are responsible for hyperthyroidism (Nagayama, Y., and Rapoport, B., "The thyrotropin receptor twenty five years after its discovery: new insights following its molecular cloning, Mol. Endocrinol. VOL.6:145-156 (1992). Autoantibodies to thyroglobulin, of uncertain pathogenetic importance, tend to be found in patients with Hashimoto's thyroiditis rather than Graves' disease.

Autoantibodies against thyroid peroxidase (TPO), however, are a sine qua non in patients with active autoimmune thyroid disease (Beever, K., Bradbury, J., Phillips, D., McLachlan, S.M., Pegg, C., Goral, A., Overbeck, W., Feifel, G., and Rees Smith, B., "Highly sensitive assays of autoantibodies to thyroglobulin and to thyroid peroxidase," Clin. Chem. VOL.35:1949-1954 (1989)). There is evidence that TPO autoantibodies play a role in thyroid cell destruction (reviewed in McLachlan, S.M., and Rapoport, B., Endocr. Rev., supra). TPO autoantibodies, which correlate well with thyroid inflammation on histological examination (Yoshida, H., Amino, N., Yagawa, K., Uemura, K., Satoh, M., Miyai, K., and Kumahara, Y., "Association of serum antithyroid antibodies with lymphocytic infiltration of the thyroid gland: Studies of seventy

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autopsied cases," *J. Clin. Endocrinol. Metab.* VOL.46:859-862
(1978)), are present in up to 25% of the adult female population
(Prentice, L.M., Phillips, D.I.W., Sarsero, D., Beever, K.,
McLachlan, S.M., and Rees Smith, B., "Geographical distribution of
subclinical autoimmune thyroid disease in Britain: a study using
highly sensitive direct assays for autoantibodies to thyroglobulin
and thyroid peroxidase," *Acta Endocrinol.* VOL.123:493-498 (1990)).
In most cases the disease is sub-clinical, because of sufficient
thyroid reserve and regeneration.

Information on the genes coding for organ-specific
autoantibodies, as well as knowledge of the autoantibody epitopes,
would be invaluable in understanding the pathogenesis of
antibody-mediated autoimmune diseases. Among the major organ-
specific human autoimmune diseases (thyroiditis, diabetes mellitus
type I, pemphigus vulgaris, myasthenia gravis, pernicious anemia
and Addison's disease), few IgG class monoclonal autoantibodies
have been produced and even fewer cloned at the molecular level,
as described in previous examples. In no disease have both the
autoantibody repertoire and the autoantigenic domains been
defined.

The present example reports the molecular cloning of the
genes for 30 new organ-specific (TPO) human autoantibodies. These
genes all code for high affinity, IgG autoantibodies. It is
demonstrated that these TPO autoantibodies, which utilize a
restricted number of H and L chain genes, encompass a restricted
immunodominant region on TPO recognized by patients with
autoimmune thyroid disease.


MATERIALS AND METHODS
Molecular cloning of TPO-binding Fab fragments: Five new human
F(ab) combinatorial cDNA libraries were constructed in the vector
Immunozap (Stratacyte, La Jolla, CA) as described in the preceding
example, with some modifications as described herein. The source

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of the mRNA for cDNA synthesis was thyroid tissue from 3 different
Graves' patients (WR, TR and JA). Two heavy (H) chain libraries
were constructed from the WR and JA cDNA, one using the "IgG1"
constant region primer of Stratacyte and the other using an IgG4
constant region primer based on nucleotide sequence data in the
IgG4 hinge region (IgG 4 Sequence Prime, 1992). In an attempt to
cover as wide a range of VH genes as possible, in addition to the
VH region primers provided by Stratacyte we used upstream primers
described by Persson et al. (Persson, M.A.A., Caothien, R.H., and
Burton, D.R., "Generation of diverse high-affinity human
monoclonal antibodies by repertoire cloning," Proc. Natl. Acad.
Sci. USA VOL.88:2432-2436 (1991)) or based on those of Marks et
al. (Marks, J.D., Tristem, M., Karpas, A., and Winter, G.,
"Oligonucleotide primers for polymerase chain reaction
amplification of human immunoglobulin variable genes and design of
family-specific oligonucleotide probes," Eur. J. Immunol. VOL.21:
985-991 (1991)).

    Preparation of soluble Fab fragments: F(ab)s were expressed
as soluble proteins in XL1-Blue cells, as described above. In
brief, protein synthesis was induced with 1 mM isopropyl-thio-
galacto-pyranoside (Sigma Chemical Co., St. Louis, MO) for 1 h at
37 C. The cells were then pelleted, frozen at -20 C, resuspended
in 0.02 volumes of 10 mM Tris pH 8.0 containing 2 ug/ml aprotinin,
1 ug/ml leupeptin, 1 ug/ml pepstatin, 0.1 mM phenylmethylsulfonyl
fluoride (all from Sigma). The suspension was sonicated,
membranes pelleted by centrifugation at 4000 x g and the F(ab)s
were affinity purified from the supernatant using a Protein G
sepharose column (Pharmacia, Piscataway, NJ).

    F(ab) binding of $^{125}$I-TPO: As described above, F(ab)s
diluted in assay buffer (0.15 M NaCl containing 10 mM Tris-HCl
pH7.5 and 0.5% bovine serum albumin) were incubated with $^{125}$I-TPO
(~ 25,000 cpm) and mouse monoclonal antibody to human kappa light
chains (QE11, Recognition Sciences, Birmingham, U.K.) in a total

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volume of 200 ul. After 1 hour at room temperature, 100 ul donkey anti-mouse Sac-cel (IDS, Boldon, Tyne and Wear, U.K.) was added, and the incubation continued for 30 minutes. After addition of 1 ml assay buffer and vortexing, the mixture was centrifuged for 5 minutes at 1000 x g to sediment the immune complexes which were then counted to determine the % radiolabeled TPO bound. The affinities of the F(ab)s for TPO were determined by Scatchard analysis from values obtained in the presence of increasing concentrations of unlabeled TPO.

Competition between F(ab)s for binding to TPO: One F(ab) was immobilized by incubation (total volume of 200 ul) with murine mAb anti-human kappa (QE11) for 1 h at room temperature. After incubation with 100 ul of Sac-cel (30 min at room temperature), the complexes were diluted in assay buffer (see above) and centrifuged at 1000 x g (5 min at 4 C). The pellets were resuspended in normal human serum diluted 1:30 in assay buffer to saturate remaining anti-kappa binding sites. In a separate set of tubes, increasing concentrations of "free" F(ab) were preincubated with $^{125}I$-TPO for 1 h at room temperature. Aliquots (100 ul) were then incubated for 30 min with the immobilized F(ab) pellets, washed with assay buffer and radioactivity bound to the Sac-cel was counted. Non-specific binding (~2% of total counts added) was subtracted to provide values for specific binding to TPO.

Competition studies between Fab fragments and serum TPO autoantibodies: Sera from 10 patients with autoimmune thyroid disease were studied. All sera contained high levels of TPO autoantibodies [detectable by ELISA (Schardt, C.W., McLachlan, S.M., Matheson, J., and Rees Smith, B., "An enzyme-linked immunoassay for thyroid microsomal antibodies," J. Immunol. Methods VOL. 55:155-168 (1982)) at dilutions of 1:1000 or greater]. Binding of $^{125}I$-TPO by serum autoantibodies was measured by precipitating the antigen-antibody complex with Protein A (Pansorbin, Calbiochem) as described above in the

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presence of increasing concentrations of Fab fragments. The Fab
fragment-TPO complex, lacking the CH2 domain of the Fc region, is
not precipitated by Pansorbin. Duplicate aliquots of sera were
incubated for 1 h at room temperature with $125_I$-TPO, alone or with
Fab fragments. Pansorbin (100 ul) was added and the incubation
continued for 30 min. After addition of 1 ml assay buffer (see
above), the mixture was vortexed, centrifuged for 30 min at 1000 x
g (4 C), supernatants removed by aspiration and TPO remaining in
the pellets counted. In preliminary experiments, serum dilutions
needed to provide binding values of ~ 20% in the absence of Fab
fragments were determined. These dilutions ranged from 1:600 –
1:2000. Non-specific $125_I$-TPO binding in the presence of control
serum without TPO antibodies was 2 – 5% of total cpm added. This
value was subtracted from the values obtained with patients' sera
in calculating the percentage inhibition by the SP Fab fragments.


RESULTS

Frequencies and subclass of TPO-specific F(ab)s: Screening 5
F(ab) combinatorial libraries from 3 new patients (WR, TR and JA)
yielded 34 TPO-binding clones which were plaque-purified. The
nucleotide sequences, determined for 32 clones, were used to
classify these clones on the basis of their presumptive germline
genes (Table VI). For completeness, TPO-specific F(ab)s from a
fourth Graves' patient, as described in preceding examples, are
also shown. The frequencies of TPO-binding F(ab)s differed
markedly between patients. Far more TPO-specific F(ab)s were
obtained from patients TR and WR than patients SP and JA.

    For two patients, two different libraries were prepared. The
WR I, TR I, JA I and the previously described SP I libraries used
an "IgG1" primer (Stratacyte, San Diego) which has since been
observed to crossprime with IgG4. The WR IV and JA IV libraries
used an IgG4 specific primer as described above. Both IgG1 and
IgG4 F(ab)s were isolated from the WR I and SP I libraries,

whereas only IgG4 F(ab)s were obtained from the WR IV library and
at a higher frequency. In contrast, the TR I library yielded only
IgGl F(ab)s. The large number of TPO-specific IgG4 F(ab)s
obtained from the WR I library and the lack of such F(ab)s from
the TR I library is consistent with the greater contribution of
Ig4 to TPO autoantibodies in the serum of patient WR relative to
patient TR.

    TPO-specific F(ab) gene usage: Analysis of the presumptive
germline genes used for 34 TPO human autoantibodies revealed the
use of relatively restricted numbers and combinations of H and L
chain genes (Table VI; Figs. 34 and 35). In particular, L chain
germline gene HUMIGKLVJ is used in TPO autoantibodies from all 4
patients. In 3 patients, HUMIGKLVJ was the only L chain obtained.
In one patient (TR), 2 other TPO-specific L chains were obtained:
A' is a VK1 (like HUMIGKLVJ) and A3 is a VK2. Of interest is an
apparent association between JK VH gene usage (see below). For
example, in 2 different patients (WR and TR), the association of
JK1 with HUMIGKLVJ is seen only with VH gene 3-1.

    The TPO-specific F(ab) H usage was less restricted than that
of the L chain. Seven different H chains were used, involving 5
VH1, 1 VH3 and 1 VH4 germline genes. The D regions were all
unique (Fig. 34). Three of the H chains (1-1, HV1L1 and 3-1) are
used in two patients. Each of these H chains is a member of the
VH1 family and is used in combination with L chain germline gene
HUMIGKLVJ. Four of the TPO-specific H chains were found only in
individual patients (4.22, 8-1B, HV1263 and V35). Of these H
chains, two were combined with L chain HUMIGKLVJ, 4.22 (a VH4) and
8-1B (a VH3). Out of a total of 7 H and L chain combinations,
only 2 were unique for both their H and L chains (HV1263/A3 and
V35/A').

    F(ab) affinities for TPO: Recombinant F(ab)s representative
of the different different H and L chain permutations were
expressed (Table VII). With the exception of clone WR4.6,

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sufficient amounts (up to ¯ 40 ug purified protein/liter) of
purified protein were obtained for further studies. Calculation
of the affinities (Kd) for human TPO revealed all F(ab)s to bind
with high affinity (¯ $10^{-10}$ M) (Table VII), comparable to
autoantibodies in patients' sera (Beever et al., (1989) supra).

Domains on TPO recognized by F(ab)s: Competition between
pairs of F(ab)s for binding to human TPO was used to define their
respective binding domains. In this approach, increasing
concentrations of one F(ab) were pre-incubated with radiolabelled
TPO and then added to a second, immobilized F(ab). Representative
experiments are shown in Fig. 36. For example (Fig. 36A), TR1.8
and TR1.9 each completely inhibited TPO binding to immobilized
TR1.9. In contrast, pre-incubation of WR1.7 and SP1.5 with
radiolabeled TPO did not prevent subsequent TPO binding to
immobilized TR1.9. Despite these differences in their ability to
compete for TPO binding to TR1.9, all free F(ab)s were capable of
binding comparable amounts of radiolabeled TPO in separate,
concurrent assays (Fig. 36B).

The above experiment (Fig. 36A and 36B) demonstrates overlap
in the areas on TPO recognized by TR1.9 and TR1.8 but not between
TR1.9 and WR1.7 or SP1.5. Similarly, there was overlap in the
WT1.7 binding domain with those of TR1.8 and SP1.5 but not with
TR1.9 (Fig. 36C). The SP1.5 binding domain overlapped that of
TR1.3 and WR1.7 but not TR1.9 (Fig. 36D). The SP1.5 domain
appeared to overlap to a small extent with that of TR1.8 (Fig.
36D). It is important to note that these differences between
F(ab) binding domains are not related to differences in their
affinities for TPO (Table VII).

Experiments covering all permutations of immobilized and free
F(ab)s suggest that there are two domains, A and B, recognized on
human TPO. The extent to which the F(ab)s interact with each
domain is summarized in Table VIII and is shown schematically in
Fig. 37. The binding sites of SP1.5 and WR4.5 lie completely

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within the TPO A domain. TR1.9 binds entirely to the B domain. TR1.8 interacts predominantly with the B domain but overlaps slightly with the A domain. Conversely, WR1.7 binds primarily to the A domain but also overlaps with TR1.8 (but not TR1.9) in the B domain. The binding site of TR1.3 spans the A and B domains equally.

Domains on TPO recognized by autoantibodies in patients' sera: The question arises as to what extent the TPO binding domains A and B reflect the binding domains of TPO autoantibodies in patients' sera. F(ab)s WR1.7 and TR1.9 were selected for competition studies with serum TPO autoantibodies because their binding sites do not overlap yet extensively cover the A and B domains. A spectrum of competition patterns was observed in 10 randomly selected sera of patients with autoimmune thyroid disease. In the representative examples shown, TPO autoantibodies in patients' sera were inhibited preferentially by WR1.7 (Fig. 38A), preferentially by TR1.9 (Fig. 38B) or in a more balanced proportion by both F(ab)s (Fig. 38C). Overall, of the 10 sera, 5 were inhibited preferentially by WR1.7, 2 by TR1.9 and 3 to approximately the same extent by WR1.7 and TR1.9. Of greater importance was the efficacy of the combination of the WR1.7 and TR1.9 F(ab)s in competing for serum TPO autoantibody binding. In the 10 sera, this combination inhibited TPO autoantibody binding by 83 $\pm$ 5 % (mean $\pm$ S.E.M.). These figures underestimate the full extent of the inhibition because of limitations to the highest concentrations of F(ab)s which could be used for competition.

DISCUSSION

The present example identifies and characterizes the genes coding for a comprensive panel of high affinity human autoantibodies to a major organ-specific autoantigen. These recombinant F(ab)s essentially cover the region on thyroid peroxidase recognized by all autoantibodies in the sera of

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patients with thyroiditis, the most common autoimmune disease.  In
the more intensively studied connective tissue diseases, genes for
numerous  autoantibodies have been characterized.  Initially,  the
"natural" and  disease-associated  autoantibodies  described  were
10     IgM,  many  of  low affinity and with polyspecificity (Casali, P.,
Inghirami, G., Nakamura, N.,  Davies,  T.F.,  and  Notkins,  A.L.,
"Human    monoclonals    from    antigen-specific    selection   of  B
lymphocytes and transformation with EBV,"   Science   VOL.234:476
(1986);  Sanz,  I.,  Casali,  P.,  Thomas, J.W., Notkins, A.L., and
15     Capra,  J.D.,  "Nucleotide  sequences  of  eight   human   natural
autoantibody  VH  regions  reveals  apparent  restricted use of VH
families," J. Immun. VOL.142:4054-4061 (1989)).   More  recently,
high  affinity  IgG-class  rheumatoid  factors  and  antibodies to
double-stranded DNA have been  produced  and  defined  (Manheimer-
20     Lory,  A.J., Davidson, A., Watkins, D., Hannigan, N., and Diamond,
B.,  "Generation and analysis of clonal IgM and  IgG  producing  B
cell  lines  expressing  an anti-DNA associated idiotype, J. Clin.
Invest. VOL.87:1519 (1991)).   However,  the  present  example  is
believed   to  constitute  the  first  definition  of  a  complete
25     autoantibody repertoire and  its  antigenic  domain(s)  in  either
organ  specific  or  non-organ specific autoimmune disease in man.
Those of  skill  will  recognize  the  diagnostic  and  especially
therapeutic  implications  of the present invention in the context
of the level of skill in  the  art,  when  the  teachings  of  the
30     present invention are fully appreciated.

       From  thyroid tissue-infiltrating B cells from three patients
with autoimmune thyroiditis, the nucleotide sequences of  30  new
TPO-specific  F(ab)s  have  been  cloned and determined.  Previous
examples present information on four antibodies  obtained  from  a
35     fourth  patient.   A limited number of H (7) and L (3) chain genes
code for this total of  34  F(ab)s.   It  is  unlikely  that  this
restriction  can  be  attributed  to  limitations  imposed  by the
primers used in the PCR to obtain the H and L chain genes.   In the

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case of the H chains, a wide range of variable region primers were
used as described above. In addition, the F(ab)s obtained used
variable regions genes from more than one VH and VK family. Most
important, the recombinant F(ab)s cover the entire antigenic
domain on TPO recognized by autoantibodies in the serum of
patients.

The most remarkable finding from analysis of the presumptive
germline genes used for TPO human autoantibodies is the relatively
restricted numbers and combinations of H and L chain genes.
Overall, among the 34 recombinant F(ab)s a total of only 7
different H and L chain combinations were found involving 7 H
chains and 3 L chains. In addition, the same combination of H and
L chains was found in three pairs of patients. These combinations
involved different H chains (HV1L1, 1-1 and 3-1) with the same L
chain (HUMIGKLVJ). The same pairing of H and L chains was
observed in murine monoclonal antibodies (specific for influenza
hemagglutinin) generated by both conventional hybridoma technology
and the combinatorial recombinant F(ab) approach (Huse, W.D.,
Sastry, L., Iverson, S.A., Kang, A.S., Alting-Mees, M., Burton,
D.R., Benkovic, S.J., and Lerner, R.A., "Generation of a large
combinatorial library of the immunoglobulin repertoire in phage
lambda," Science VOL.246:1275-1281 (1989). Further, our F(ab)s
had very high affinities for TPO ($~10^{-10}$M), comparable with those
of serum TPO autoantibodies (Beever et al., (1989) supra).
Studies of "natural" autoantibodies and autoantibodies to
erythrocyte antigens have shown over-representation of VH4 family
genes (Sanz et al., (1989) supra; Capra, J.D., Scand. J. Immunol
(1992)). In contrast, 5/7 of our TPO-specific F(ab) combinations
were derived from VH1 family germline genes. The other 2 F(ab)
utilized putative VH3 and VH4 germline genes. Among the
VH1-encoded TPO F(ab)s, the most abundant were related to the
recently described HV1L1 germline gene, which is utilized in a
rheumatoid factor (Carson, D., HV1L1. J. Exp. Med (1992)).

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Further, the 1-1 germline gene apparently used by two other TPO F(ab)s ranks very close to BV1L1 in the VH1 family. Because of consistent amino acid differences between the TPO F(abs) described here and BV1L1, it is likely that the former are not derived from BV1L1 but from another, as yet undescribed, germline gene.

There is evidence that the ability to produce TPO autoantibodies is inherited as an autosomal dominant trait in women with incomplete penetrance in men (Phillips, D., McLachlan, S., Stephenson, A., Roberts, D., Moffitt, S., McDonald, D., Ad'Eiah, A., Stratton, A., Young, E., Clark, F., Beever, K., Bradbury, J., and Rees-Smith, B., "Autosomal dominant transmission of autoantibodies to thyroglobulin and thyroid peroxidase," *J. Clin. Endocrinol. Metab.* VOL.70:742-74 (1990); Phillips, D., Prentice, L., Upadhyaya, M., Lunt, P., Chamberlain, S., Roberts, D.F., McLachlan, S., and Rees Smith, B., "Autosomal dominant inheritance of autoantibodies to thyroid peroxidase and thyroglobulin -- Studies in families not selected for autoimmune thyroid disease," *J. Clin. Endocrinol. Metab.* VOL. 72:973-975 (1991)). The location and nature of the gene or gene cluster responsible for this inheritance are unknown. Polymorphisms at the VH locus are associated with autoantibody production (Olee, T., Yang, P-M., Siminovitch, K.A., Olsen, N.J., Hillson, J., Wu, J., Kozin, F., Carson, D.A., and Chen, P.P., "Molecular basis of an autoantibody-associated restriction fragment length polymorphism that confers susceptibility to autoimmune diseases," *J. Clin. Invest.* VOL.88:193-203, (1991); Shin, E.K., Matsuda, F., Nagaoka, H., Fukita, Y., Imai, T., Yokoyama, K., Soeda, E., and Honjo, T., "Physical map of the 3' region of the human immunoglobulin heavy chain locus: clustering of autoantibody-related variable segments in one haplotype, *EMBO J.* VOL.10:3641-3645 (1991)). Comparison of the Figures of the present example shows that one of the TPO F(ab)s described here is encoded by a VH gene involved in one of these polymorphisms. Knowledge of the

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genetic background of TPO-specific autoantibodies may provide insight into the basis for the inheritance of thyroid autoimmunity, and will provide a basis for the development of relevant diagnostic and therapeutic compositions and methods.

TPO, the primary autoantigen in autoimmune thyroiditis, is a glycoprotein expressed on the surface of the thyroid follicular cells (reviewed in McLachlan and Rapoport, 1992). TPO autoantibodies in patients' sera are heterogeneous and the majority recognize a conformational epitope(s),(Gardas, A., and Domek, H., "The effect of sulphydryl reagents on the human thyroid microsomal antigen," *J. Endocrinol. Invest.* VOL.11:385-388 (1988); Nakajima, Y., Howells, R.D., Pegg, C., Davies Jones, E., and Rees Smith, B., "Structure activity analysis of microsomal antigen/thyroid peroxidase," *Molec. Cell. Endocrinol.* VOL. 53:15-23 (1987); Portolano et al.1992). Despite studies using different approaches (reviewed in McLachlan and Rapoport (1992) _supra_), the number of epitopes recognized by TPO autoantibodies, or even the number of antigenic domains involved, has been unknown.

Using a comprehensive repertoire of recombinant human F(ab)s, the present invention defines two major epitopic areas, A and B, on TPO. Essentially all serum TPO autoantibodies interact with the A and B domains. TPO is a large (933 amino acid, 107 kD) (Magnusson, R., Chazenbalk, G., Gestautas, J., Seto, P., Filetti, S., and Rapoport, B., "Sequences of interest: molecular cloning of the complementary deoxyribonucleic acid for human thyroid peroxidase," *Mol. Endocrinol.* VOL.1:856-861 (1987)) globular protein relative to the size of the F(ab) binding site (Davies, D.R., and Padlan, E., "Antibody-Antigen Complexes," *Annu. Rev. Biochem.* VOL.59: 439-47 (1990)). For this reason and because a combination of only two different Fabs can compete for binding by all TPO autoantibodies, the autoimmunogenic region comprises a single, relatively small area (domains A + B). As described above, it is possible that serum TPO autoantibodies to other

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regions of TPO, including linear epitopes, exist, but these will comprise a very small proportion of the repertoire.

The present invention now permits analysis of the relationship between H and L chain genes and the antigenic domains on TPO recognized by TPO autoantibodies. Such an analysis has not previously been possible in an autoimmune disease. The clearest association is between the L chain gene HUMIGKLVJ and the A domain on TPO. In contrast, the B domain is most closely associated with the L chains A' and A3. The H chains are more diverse and may alter the fine specificity of the F(ab) binding site by interacting with antigenic areas adjacent to the L chain binding site. In some instances, exemplified by TR1.3 (VH 8-1b/ VK HUMIGKLVJ), the H chain shifts the F(ab) binding from the A domain to cover both A and B domains.

Of interest is the observation that F(ab)s of both subclass IgG1 and IgG4 can interact with the A domain on TPO. Among these are F(ab)s encoded for by two closely related VHI genes, namely 1-1 in the case of IgG1 F(ab)s and HVIL1 in the case of IgG4 F(ab)s. This apparent difference in germline gene origin suggests that the IgG4 F(ab)s are not derived from the IgG1 F(ab)s by subclass switching. In rheumatoid factors, the same germline gene has clearly been shown to code for a low affinity IgM and subsequently for an affinity-matured IgG molecule (Randen, I., Brown, D., Thompson, K.M., Hughes-Jones, N., Pascual, V., Victor, K., Capra, J.D., Forre, O., and Natvig, J.B., "Clonally related IgM rheumatoid factors undergo affinity maturation in the rheumatoid synovial tissue," *J. Immun.* VOL.148:3296-3301 (1992)). In constrast, observations of crossreactive idiotypes in human antibodies to H. influenzae b polysaccharide indicate independent B cell lineages in IgG1 and IgG2 antibodies (Lucas, A.H., and Granoff, D.M., "A major crossreactive idiotype associated with human antibodies to the Haemophilus influenza b polysaccharide. Expression in relation to age and immunoglobulin G subclass," *J.*

*Clin. Invest.* **VOL.85**: 1158-1166 (1990)). However, as mentioned above, it is possible that HV1L1 is not the germline gene from which the IgG4 TPO-specific F(ab)s were derived. Therefore, we cannot exclude the possibility that both IgG1 and IgG4 TPO-specific F(ab)s are derived by switching from the same, as yet undescribed, VH1 germline gene.

Studies in mice have provided evidence for (Shlomchik, M.J., Marshak-Rothstein, A., Wolfowicz, C.B., Rothstein, T.L., and Weigert, M.G., "The role of clonal selection and somatic mutation in autoimmunity," *Nature* **VOL.328**:805-811 (1987); O'Keefe, T.L., Bandyopadhyay, S., Datta, S.K., and Imanishi-Kari, T., "V region sequences of an idiotypically connected family of pathogenic anti-DNA autoantibodies," *J. Immun.* **VOL.** 144:4275-4283 (1990); Eilat, D., and Fischel, R., "Recurrent utilization of genetic elements in V regions of antinucleic acid antibodies from autoimmune mice," *J. Immun.* **VOL.**147:361-368 (1991)) and against (Panosian-Sahakian, N., Klotz, J.L., Ebling, F., Kronenberg, M., and Hahn, B., "Diversity of Ig V gene segments found in anti-DNA autoantibodies from a single (NZB x NZW)F1 mouse," *J. Immun.* **VOL.**142:4500-4506 (1989)) restricted H and L chain usage in rheumatoid factors and DNA autoantibodies. Similarly, a diverse array of immunoglobulin genes occurs in IgM-class rheumatoid factors (Pascual, V., Victor, K., Randen, I., Thompson, K., Natvig, J.B., and Capra, J.D. "IgM rheumatoid factors in patients with rheumatoid arthritis derive from a diverse array of germline immunoglobulin genes and display little evidence of somatic variation," *J. Rheum.*, (1992)). A possible explanation for these divergent observations is that the autoantibodies are interacting with a wide range of epitopes. For example, thyroglobulin autoantibodies, induced by immunization in mice, interact with a wide range of epitopes and are derived from a large number of V region gene segments (Gleason, S.L., Gearhart, P., Rose, N.R., and Kuppers, R.C., "Autoantibodies to thyroglobulin are encoded by V-gene segments and recognize

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restricted epitopes," *J. Immun.* VOL.145:1768-1775 (1990)). The limited antigenic region on TPO recognized by disease-associated autoantibodies simplifies this issue. Under these conditions, there is, indeed, restriction in H and L chain usage in human, organ-specific autoimmune disease.

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| Library (Frequency) | Subclass | Clone | H Chain | | | L Chain | | |
|---|---|---|---|---|---|---|---|---|
| | | | Germline[a] VH | JH | | Germline[a] VK | JK | |
| WR I (11/90,000) | 1 | 1.7; 1.9 | 3-1 | 1 | 4 | KLVJ | 1 | 1 |
| | 4 | 4.2-5; 4.8 4.10-12 | HV1L1 | 1 | 4 | KLVJ | 1 | 2 |
| | 4 | 4.6 | 4.22 | 4 | 5 | KLVJ | 1 | 2 |
| WR IV (15/30,000) | 4 | 4.21; 4.22; 4.25-35 | HV1L1 | 1 | 4 | KLVJ | 1 | 2 |
| TR I (7/90,000) | 1 | 1.3; 1.5 | 8-1B | 3 | 4 | KLVJ | 1 | 2 |
| | 1 | 1.6; 1.8 | HV1263 | 1 | 3 | A3 | 2 | 2 |
| | 1 | 1.9; 1.13 | V35 | 1 | 4 | A' | 1 | 4 |
| | 1 | 1.10 | 3-1 | 1 | 4 | KLVJ | 1 | 1 |
| JA I (1/200,000) | 1 | 1.9 | 1-1 | 1 | 6 | KLVJ | 1 | 4 |
| JA IV (0/200,000) | - | - | | | | | | |
| SP I[b] (3/180,000) | 1 | 1.2 | 1-1 | 1 | 6 | KLVJ | 1 | 2 |
| | | 1.4; 1.5 | 1-1 | 1 | 6 | KLVJ | 1 | 1 |
| | 4 | 4.6 | HV1L1 | 1 | 4 | KLVJ | 1 | 2 |

Table VI: Summary of TPO-specific human F(ab)s obtained from 4 Graves' patients. Combinatorial libraries were constructed from mRNA prepared from intrathyroidal B cells as described. a) Presumptive germline genes. b) Previously published. Clone SP4.6 was obtained by recombining the SP1.2 L chain with the parent SP I H chain library as described herein.

| H Chain | L Chain | Clone | Affinity (Kd) |
|---------|---------|-------|---------------|
| HV1L1   | KLVJ    | WR4.5 | 3.0, 3.2 $\times 10^{-10}$ M |
|         |         | SP4.6[b] | 1.0 ± 0.2 $\times 10^{-10}$ M |
| 3-1     | KLVJ    | WR1.7 | 1.2, 2.9 $\times 10^{-10}$ M |
|         |         | TR1.10 | 1.7, 1.4 $\times 10^{-10}$ M |
| 8-1B    | KLVJ    | TR1.3 | 5.1 ± 0.1 $\times 10^{-10}$ M |
| 1-1     | KLVJ    | SP1.2[a] | 8.0 ± 1.0 $\times 10^{-11}$ M |
|         |         | SP1.4[a] | 2.0; 2.0 $\times 10^{-10}$ M |
|         |         | SP1.5[a] | 8.0 ± 1.0 $\times 10^{-11}$ M |
| HV1263  | A3      | TR1.8 | 2.7 ± 0.1 $\times 10^{-10}$ M |
| V35     | A'      | TR1.9 | 1.5 ± 0.2 $\times 10^{-10}$ M |

Table VII: Affinities for human TPO of expressed F(ab) fragments.
Values from duplicate or triplicate experiments were calculated by
Scatchard analysis. [a]; [b]Previously described herein.

| Clone | TPO Domains | | H Chain | L Chain |
|-------|---|---|---------|---------|
|       | A | B |         |         |
| SP1.5 | ++++ | - | 1-1 | KLVJ |
| WR4.5 | ++++ | - | HV1L1 | KLVJ |
| WR1.7 | ++++ | ++ | 3-1 | KLVJ |
| TR1.3 | ++++ | ++++ | 8-1B | KLVJ |
| TR1.8 | + | ++++ | HV1263 | A3 |
| TR1.9 | - | ++++ | V35 | A' |

Table VIII: Domains on TPO recognized by F(ab)s as determined by competition between pairs of F(ab)s for antigen binding. "++++" Represents complete and "-" represents no overlap with the indicated domain. "+" or "++" indicate partial overlap.

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BRIEF DESCRIPTION OF THE DRAWINGS

(Continued from Page 16)

Fig. 21:   Binding affinity of Fab fragment SP2 for recombinant human thyroid peroxidase (TPO).  Brackets indicate the mean $\pm$ the range of duplicate densitometric values obtained for each TPO concentration in a representative experiment.  Comparable results were obtained in two additional experiments.

Fig. 22:   Nucleotide and derived amino acid sequence of the SP2 heavy chain compared with the VHI germline gene 1-1 and the JH3 germline gene (Pascual, V., and Capra, J.D. Adv. Immunol. VOL.49:1-74 (1991)).  The complementarity determining regions (CDR) are indicated by the asterisks.  Identical nucleotides are depicted by dashes and deleted nucleotides by dots.

Fig. 23:  Nucleotide and derived amino acid sequence of the SP2 light chain compared with the VKI germline gene HUMIGKLVJ (GenBank accession number D90158) and the JK2 germline gene in Taykv312 (Kipps, T.J., Tomhave, E., Chen, P.P., and Fox, R.I. J.Immun. VOL.142:4261-4268 (1989)).  The complementarity determining regions (CDR) are indicated by the asterisks.  Identical nucleotides are depicted by dashes.

Figure 24:  Nucleotide sequences of the VK regions of TPO Fab fragments SP1.2, SP1.4, SP1.5 and the VK1 germline gene HUMIGKLVJ (GenBank accession number D90158) together with the consensus sequence for all 4 genes.  Identical nucleotides are indicated by dots.  The nucleotides at positions 3 and 4, which differ from the germline gene sequence, are mutations required to introduce the Sac I restriction site used to clone the light chain genes.  The

complementarity-determining regions (CDR) 1, 2 and 3 are indicated by asterisks.

Figure 25: Derived amino acid sequence (in single-letter code) of VK regions of TPO Fab fragments SP1.2, SP1.4, SP1.5 and HUMIGKLVJ (GenBank accession number D90158) together with the consensus sequence. Identical residues are indicated by dots and CDR1, CDR2 and CDR3 are indicated by asterisks. The amino acid residues at positions 1 and 2, which differ from the germline gene, are encoded by the Sac I restriction site introduced for cloning the light chain genes.

Figure 26: Nucleotide and derived amino acid sequences for the JK regions of TPO Fab fragments SP1.2, SP1.4, SP1.5, the germline gene J-KV312 (Kipps, T.J., E. Tomhave, P.P. Chen, and R.I. Fox, "Molecular characterization of a major autoantibody-associated cross-reactive idiotype and Sjogren's Syndrome," J. Immun., VOL. 142: 4261-4268 (1989)) and the consensus sequence. Identity of residues is indicated by dots.

Figure 27: A. Effect of increasing molar (M) concentrations of TPO, lactoperoxidase (LPO) or myeloperoxidase (MPO) on the binding of $^{125}I$-TPO by SP1.2. Background binding in the absence of Fab fragments ( ̄ 2%) was subtracted. B. Competition inhibition by unlabeled TPO of radiolabeled TPO binding to the FAB fragments. In the absence of unlabeled TPO, binding values for the three Fab fragments were 13-15%. Background of ̄ 2% was subtracted. Dissociation constants (Kd) were determined by Scatchard analysis (Scatchard, G., "The attractions of proteins for small molecules and ions," Ann. NY Acad. Sci. VOL 51: 660-672 (1949)) and are:

SP2 = $8.3 \times 10^{-11}$M; SP4 = $2.2 \times 10^{-10}$M; SP5 = $6.3 \times 10^{-11}$M.

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Figure 28: Inhibition by increasing molar (M) concentrations of
SP1.2 on the binding to $^{125}$I-TPO by serum TPO autoantibodies. The
mean values ($\pm$ S.E.M.) obtained for sera from 11 patients are
shown by solid circles. Background binding by serum from a TPO
autoantibody negative donor was not subtracted and is shown by the
open circles (mean $\pm$ S.E.M. of 3 experiments).

Figure 29: Competition ELISA for binding to TPO between the
SP1.2 Fab fragment and TPO autoantibodies of different IgG
subclasses. Panels A, B and C show data obtained with three
different patients. TPO autoantibody levels are shown as the O.D.
readings measured at 492nm. Background O.D. values obtained for
TPO autoantibody-negative serum were <0.05. SP1.2 (M); molar
concentration of SP1.2.

Figure 30: Effect of denaturation of TPO on SP Fab fragment
binding. Binding of SP1.2, SP1.4 and SP1.5 (left panel) or mouse
monoclonal antibody #40.28 (right panel) was measured to native or
denatured TPO by ELISA. Binding is shown as the O.D. value at
492nm. Background O.D. values for TPO autoantibody negative serum
and control murine ascites were <0.05.

Figure 31: A) Nucleotide sequence of the VH region of F(ab) SP4.6
compared with the closest germline gene, hvlL1 (Olee et al). B)
Derived amino acid sequence of the VH region of SP4.6, again in
comparison with hvlL1. C) Nucleotide and derived amino acid
sequences of the SP4.6 D region. In panels A and B, dashes
represent differences from the consensus sequence. Dots represent
identical nucleotides. Complementarity determining regions (CDR)
are boxed. Amino acids are shown in the single letter code.
Residue numbering is according to Kabat et al. (Kabat, E.A., T.T.
Wu, H.M. Perry, K.S. Gottesman, and C. Foeller, "Sequences of

proteins of immunological interest. U.S. Department of Health and Human Services," (1991)).

Figure 32: A) Derived amino acid sequences of V kappa regions of anti-TPO F(ab)s SP1.12 through SP1.22 compared with the closest germline gene, HUMIGKLVJ (GenBank Accession number D90158). B) Derived amino acid sequences of the J kappa regions of SP1.12 through SP1.22. Dashes represent differences from the consensus sequence. Dots represent identical nucleotides. Complementarity determining regions (CDR) are boxed.

Figure 33: Binding domains on TPO for the SP1.2, SP4.6, SP1.20 F(ab)s. $^{125}$I-TPO was preincubated in the absence or presence of increasing concentrations of SP4.6, SP1.20 or SP1.2 [Free F(ab)]. The ability of these complexes to bind to immobilized SP1.2 was then determined. The results are expressed as % 125I-TPO bound after subtraction of background values (~ 2%) obtained using buffer alone.

Figure 34: Derived amino acid sequences of TPO-specific F(ab) H chains. Designation of the framework (FR) and complementarity determining regions (CDR) is according to Kabat (Kabat, E.A., Wu, T.T., Perry, H.M., Gottesman, K.S., and Foeller, C., "Sequences of proteins of immunological interest," *U.S. Department of Health and Human Services* (1991)) and Pascual and Capra [74]: 8-1B, 3-1 (Kabat et al.1991)
a) Same as WR4.3,4,5,8,11,21,22,33,36,37 with the exception that WR4.3,5 and 8 have Q (for E) at position 7.
b) Same as SP1.4,5 [Portolano et al.1992).
c) Same as TR1.13
The Q to K substitution at position 3 represents the restriction site introduced by the PCR oligonucleotide. The presence or

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absence of an E at position 6 reflects the use of different PCR
primers.


Figure 35: Derived amino acid sequences of TPO-specific F(ab) L
chains. Designation of the framework (FR) and complementarity
determining regions (CDR) and the JK germline genes are according
to Kabat et al (1991). The sources of the VK germline genes are:
-HUMIGKLVJ (Genbank accession number D90158); A' and A3 (Kabat et
al.1991).
a) Same as WR4.5
b) Same as WR4.31
c) Same as WR.K27,28,34                                           :


Figure 36: Domains on TPO recognized by F(ab)s. Increasing
concentrations of one F(ab) were pre-incubated with radiolabelled
TPO and then added to a second, immobilized F(ab) (Methods). The
immobilized F(ab) was TR1.9 (panel A), TR1.7 (panel C) and SP1.5
(panel D).


The ability of the free F(ab) to inhibit binding to itself is
shown by the open circles. Confirmation of the binding potency of
the free F(ab)s was determined concurrently in each experiment. A
representative control (panel B for the experiment in panel A) is
shown.


Figure 37: Schematic representation of the binding domains on TPO
for the
expressed F(ab)s.


Figure 38: Domains on TPO recognized by autoantibodies in 3
representative sera (panels A, B and C) from patients with
autoimmune thyroid disease. F(ab)s WR1.7 and TR1.9, alone or in

5

combination, were used to compete for serum autoantibody binding
to radiolabeled TPO (Methods).

## CLAIMS

**WHAT IS CLAIMED IS:**

1. Recombinant, enzymatically active, human thyroid peroxidase, or a functional or chemical derivative thereof.

2. The human thyroid peroxidase of claim 1 produced by non-thyroidal eukaryotic cells.

3. A plasmid selected from the group consisting of pECE-HTPO, pHTPO(M1)-ECE-SV2-DHFR, pHTPO-DHFR-2B, pHTPO-DHFR-4C and pHTPO-DHFR-4C-MTX.

4. A non-thyroidal eukaryotic cell transformed with the plasmid of claim 3.

5. A method of producing human thyroid peroxidase, comprising culturing the transformed cell of claim 4 under conditions allowing expression of human thyroid peroxidase, and recovering said human thyroid peroxidase.

6. An antibody against the human thyroid peroxidase of claim 1.

7. A method of detecting human thyroid peroxidase in a sample comprising contacting said sample with the antibody of claim 6, wherein said antibody is detectably labeled, so as to form a complex between the human thyroid peroxidase in said sample and said detectably labeled antibody, and detecting the complexed or uncomplexed detectably labeled antibody.

8. A kit for the detection of human thyroid peroxidase in a sample, comprising container means comprising one or more

containers, wherein one of said containers comprises the
antibody of claim 6, wherein said antibody is detectably
labeled.

5      9.   A method of detecting an antibody to human thyroid
peroxidase in a sample, comprising contacting a sample
suspected of having an antibody to human thyroid peroxidase
with the recombinant human thyroid peroxidase of claim 1,
wherein said recombinant human thyroid peroxidase is
10     detectably labeled, so as to form a complex between said
antibody to human thyroid peroxidase present in said sample
and said detectably labeled recombinant human thyroid
peroxidase, and detecting the complexed or uncomplexed
detectably labeled recombinant human thyroid peroxidase.

15
       10.  A kit for the detection of an antibody to human
thyroid peroxidase in a sample, comprising container means
comprising one or more containers, wherein one of said
containers comprises the recombinant human thyroid peroxidase
20     of claim 1, wherein said recombinant human thyroid peroxidase
is detectably labeled.

       11.  A recombinant DNA sequence encoding human thyroid
peroxidase which is secreted from a cell.

25
       12.  The DNA sequence of claim 11 wherein said sequence
possesses a stop codon upstream from a transmembrane domain.

       13.  The DNA sequence of claim 12 wherein said sequence
30     possesses a stop codon upstream from nucleotides encoding
amino acid residues 846-870 as shown in figure 7.

       14.  A vector which comprises the DNA sequence of claim
11, 12 or 13.

15. A host cell transformed with the vector of claim 14.

16. Human thyroid peroxidase produced by the host cell
of claim 15, or a functional or chemical derivative thereof.

17. A method of producing human thyroid peroxidase,
comprising culturing the host cell of claim 15 under
conditions allowing the expression and secretion of secretable
human thyroid peroxidase, and recovering said human thyroid
peroxidase.

18. An antibody against the human thyroid peroxidase of
claim 16.

19. A method of detecting human thyroid peroxidase in a
sample, comprising contacting a sample suspected of having
human thyroid peroxidase with the antibody of claim 18,
wherein said antibody is detectably labeled, so as to form a
complex between said human thyroid peroxidase present in said
sample and said detectably labeled antibody, and detecting the
complexed or uncomplexed detectably labeled antibody.

20. A kit for the detection of human thyroid peroxidase
in a sample, comprising container means comprising one or more
containers, wherein one of said containers comprises the
antibody of claim 18, wherein said antibody is detectably
labeled.

21. A method of detecting an antibody to human thyroid
peroxidase in a sample, comprising contacting a sample
suspected of having an antibody to human thyroid peroxidase
with the recombinant human peroxidase of claim 16, wherein
said recombinant human thyroid peroxidase is detectably

labeled, so as to form a complex between said antibody to human thyroid peroxidase present in said sample and said detectably labeled recombinant human thyroid peroxidase, and detecting the complexed or uncomplexed detectably labeled

5  recombinant human thyroid peroxidase.

22.  A kit for the detection of an antibody to human thyroid peroxidase in a sample, comprising container means comprising one or more containers, wherein one of said

10  containers comprises the recombinant human thyroid peroxidase of claim 16, wherein said recombinant human thyroid peroxidase is detectably labeled.

23.  A recombinant human thyroid peroxidase peptide

15  comprised of the nine amino acid region comprising residues 713-721 of the thyroid peroxidase of claim 1.

24.  Recombinant thyroid peroxidase wherein a nine amino acid region comprising residues 713-721 have been deleted or

20  replaced.

25.  A peptide capable of binding the B-cell epitope on thyroid peroxidase.

25  26.  The peptide of claim 25, wherein said peptide is isolated from a B-cell.

27.  The B-cell protein of claim 26, wherein said protein is recombinantly produced.

30  28.  The DNA sequence encoding the peptides of claim 25, 26, or, 27.

29. An antibody against the recombinant thyroid peroxidase peptide of claim 23.

30. The antibody of claim 29 wherein said antibody is selected from the group consisting of a monoclonal antibody, polyclonal antibody, an anti-idiotypic antibody, or an anti anti-idiotypic antibody.

31. A method of detecting human thyroid peroxidase in a sample comprising contacting said sample with the antibody of claim 29, wherein said antibody is detectably labeled, so as to form a complex between the human thyroid peroxidase in said sample and said detectably labeled antibody, and detecting the complexed or uncomplexed detectably labeled antibody.

32. A pharmaceutical preparation comprising the anti-idiotypic antibody of claim 30.

33. A method of treating Hashimoto's thyroiditis comprising the pharmaceutical preparation of claim 32.
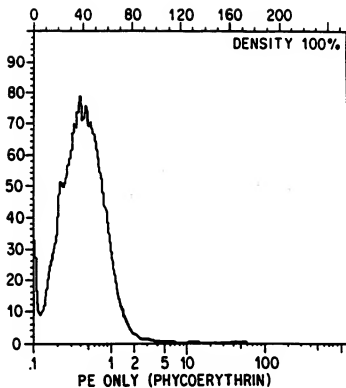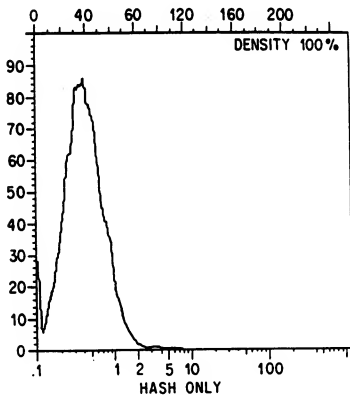
FIG. 2A



FIG. 2B
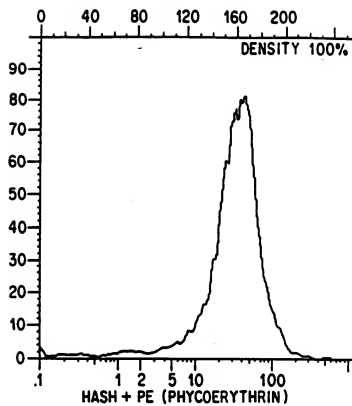
SUBSTITUTE SHEET

*FIG. 2C*
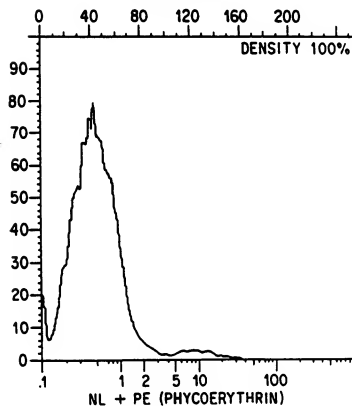
*FIG. 2D*
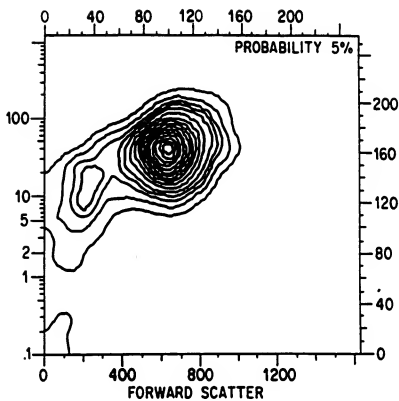
FIG. 2E

HASH + PE
(PHYCOERYTHRIN)



FIG. 2F

NL + PE
(PHYCOERYTHRIN)
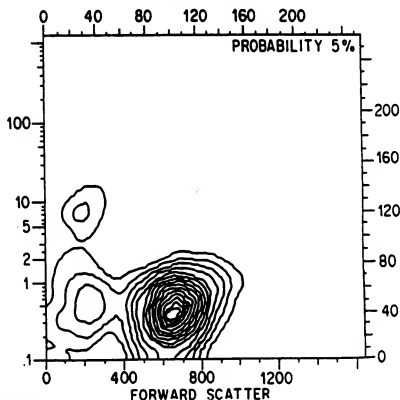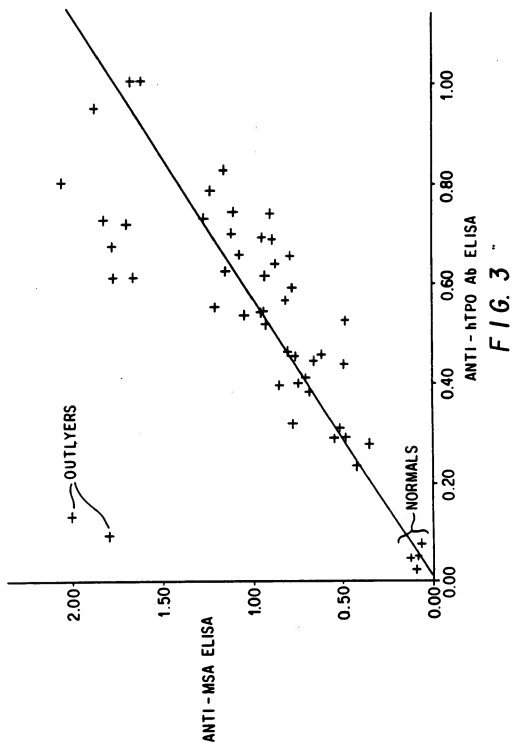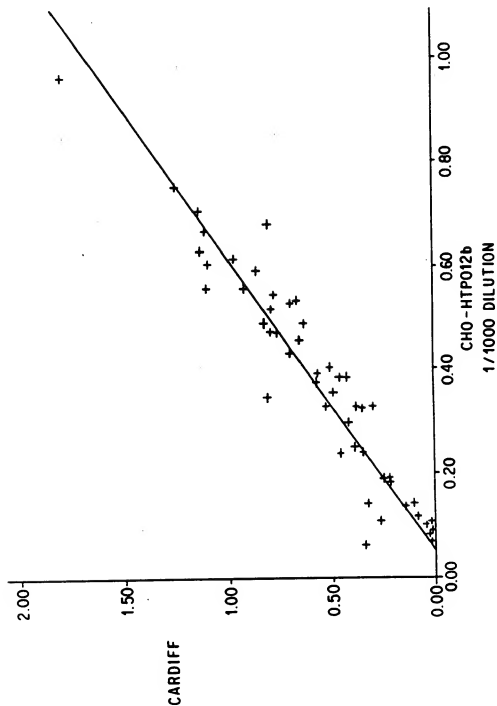
*F I G. 3*

CHO-HTP012b
1/1000 DILUTION

CARDIFF

*FIG. 4*
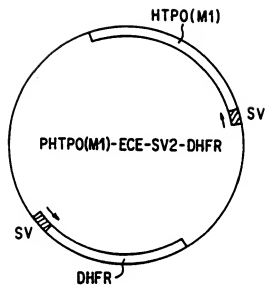
FIG. 8
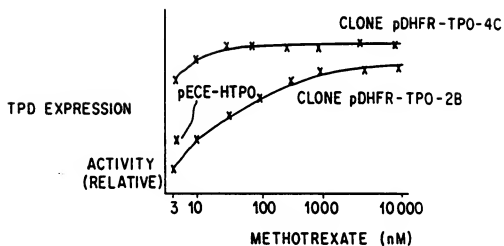


FIG. 5

2611

AGG CTC CCT CGG GTG ACT TGG ATC TCC ATC ATG TCG CTG GCT GCT CTG CTG ATC G    pHTPD-BS    2662

Eco R1

Stop    Stop

AGG CTC CCT CGG GTG ACT TGA ATT CCC ATG TAG CTG GCT GCT CTG CTG ATC G    pHTPD(H1)-BS

Stop    Stop

*F I G. 6*

```
                                   27                                    54
GAG GCA ATT GAG GCG CCC ATT TCA GAA GAG TTA CAG CCG TGA AAA TTA CTC AGC
                                    •   • •••            •  •••

                                   81                                    108
AGT GCA GTT GGC TGA GAA GAG GAA AAA AGA ATG AGA GCG CTG GCT GTG CTG TCT
                                            MET Arg Ala Leu Ala Val Leu Ser

                                   135                                   162
GTC ACG CTG GTT ATG GCC TGC ACA GAA GCC TTC TTC CCC TTC ATC TCG AGA GGG
Val Thr Leu Val Met Ala Cys Thr Glu Ala Phe Phe Pro Phe Ile Ser Arg Gly

                                   189                                   216
AAA GAA CTC CTT TGG GGA AAG CCT GAG GAG TCT CGT GTC TCT AGC GTC TTG GAG
Lys Glu Leu Leu Trp Gly Lys Pro Glu Glu Ser Arg Val Ser Ser Val Leu Glu

                                   243                                   270
GAA AGC AAG CGC CTG GTG GAC ACC GCC ATG TAC GCC ACG ATG CAG AGA AAC CTC
Glu Ser Lys Arg Leu Val Asp Thr Ala Met Tyr Ala Thr Met Gln Arg Asn Leu

                                   297                                   324
AAG AAA AGA GGA ATC CTT TCT GGA GCT CAG CTT CTG TCT TTT TCC AAA CTT CCT
Lys Lys Arg Gly Ile Leu Ser Gly Ala Gln Leu Leu Ser Phe Ser Lys Leu Pro

                                   351                                   378
GAG CCA ACA AGC GGA GTG ATT GCC CGA GCA GCA GAG ATA ATG GAA ACA TCA ATA
Glu Pro Thr Ser Gly Val Ile Ala Arg Ala Ala Glu Ile Met Glu Thr Ser Ile

                                   405                                   432
CAA GCG ATG AAA AGA AAA GTC AAC CTG AAA ACT CAA CAA TCA CAG CAT CCA ACG
Gln Ala Met Lys Arg Lys Val Asn Leu Lys Thr Gln Gln Ser Gln His Pro Thr

                                   459                                   486
GAT GCT TTA TCA GAA GAT CTG CTG AGC ATC ATT GCA AAC ATG TCT GGA TGT CTC
Asp Ala Leu Ser Glu Asp Leu Leu Ser Ile Ile Ala Asn Met Ser Gly Cys Leu
                                                              X
```

## FIG. 7A

```
                        513                                    540
CCT TAC ATG CTG CCC CCA AAA TGC CCA AAC ACT TGC CTG GCG AAC AAA TAC AGG
Pro Tyr Met Leu Pro Pro Lys Cys Pro Asn Thr Cys Leu Ala Asn Lys Tyr Arg

                        567                                    594
CCC ATC ACA GGA GCT TGC AAC AAC AGA GAC CAC CCC AGA TGG GGC GCC TCC AAC
Pro Ile Thr Gly Ala Cys Asn Asn Arg Asp His Pro Arg Trp Gly Ala Ser Asn

                        621                                    648
ACG GCC CTG GCA CGA TGG CTC CCT CCA GTC TAT GAG GAC GGC TTC AGT CAG CCC
Thr Ala Leu Ala Arg Trp Leu Pro Pro Val Tyr Glu Asp Gly Phe Ser Gln Pro

                        675                                    702
CGA GGC TGG AAC CCC GGC TTC TTG TAC AAC GGG TTC CCA CTG CCC CCG GTC CGG
Arg Gly Trp Asn Pro Gly Phe Leu Tyr Asn Gly Phe Pro Leu Pro Pro Val Arg

                        729                                    756
GAG GTG ACA AGA CAT GTC ATT CAA GTT TCA AAT GAG GTT GTC ACA GAT GAT GAC
Glu Val Thr Arg His Val Ile Gln Val Ser Asn Glu Val Val Thr Asp Asp Asp

                        783                                    810
CGC TAT TCT GAC CTC CTG ATG GCA TGG GGA CAA TAC ATC GAC CAC GAC ATC GCG
Arg Tyr Ser Asp Leu Leu MET Ala Trp Gly Gln Tyr Ile Asp His Asp Ile Ala

                        837                                    864
TTC ACA CCA CAG AGC ACC AGC AAA GCT GCC TTC GGG GGA GGG TCT GAC TGC CAG
Phe Thr Pro Gln Ser Thr Ser Lys Ala Ala Phe Gly Gly Gly Ser Asp Cys Gln

                        891                                    918
ATG ACT TGT GAG AAC CAA AAC CCA TGT TTT CCC ATA CAA CTC CCG GAG GAG GCC
Met Thr Cys Glu Asn Gln Asn Pro Cys Phe Pro Ile Gln Leu Pro Glu Glu Ala

                        945                                    972
CGG CCG GCC GCG GGC ACC GCC TGT CTG CCC TTC TAC CGC TCT TCG GCC GCC TGC
Arg Pro Ala Ala Gly Thr Ala Cys Leu Pro Phe Tyr Arg Ser Ser Ala Ala Cys
```

## FIG. 7B

```
                                 999                                    1026
GGC ACC GGG GAC CAA GGC GCG CTC TTT GGG AAC CTG TCC ACG GCC AAC CCG AGG
Gly Thr Gly Asp Gln Gly Ala Leu Phe Gly Asn Leu Ser Thr Ala Asn Pro Arg
                                                     X

                                 1053                                   1080
CAG CAG ATG AAC GGG TTG ACC TCG TTC CTG GAC GCG TCC ACC GTG TAT GGC AGC
Gln Gln Met Asn Gly Leu Thr Ser Phe Leu Asp Ala Ser Thr Val Tyr Gly Ser

                                 1107                                   1134
TCC CCG GCC CTA GAG AGG CAG CTG CGG AAC TGG ACC AGT GCC GAA GGG CTG CTC
Ser Pro Ala Leu Glu Arg Gln Leu Arg Asn Trp Thr Ser Ala Glu Gly Leu Leu .
                                                 X

                                 1161                                   1188
CGC GTC CAC GGC CGC CTC CGG GAC TCC GGC CGC GCC TAC CTG CCC TTC GTG CCG
Arg Val His Gly Arg Leu Arg Asp Ser Gly Arg Ala Tyr Leu Pro Phe Val Pro

                                 1215                                   1242
CCA CGC GCG CCT GCG GCC TGT GCG CCC GAG CCC GGC AAC CCC GGA GAG ACC CGC
Pro Arg Ala Pro Ala Ala Cys Ala Pro Glu Pro Gly Asn Pro Gly Glu Thr Arg

                                 1269                                   1296
GGG CCC TGC TTC CTG GCC GGA GAC GGC CGC GCC AGC GAG GTC CCC TCC CTG ACG
Gly Pro Cys Phe Leu Ala Gly Asp Gly Arg Ala Ser Glu Val Pro Ser Leu Thr

                                 1323                                   1350
GCA CTG CAC ACG CTG TGG CTG CGC GAG CAC AAC CGC CTG GCC GCG GCG CTC AAG
Ala Leu His Thr Leu Trp Leu Arg Glu His Asn Arg Leu Ala Ala Ala Leu Lys

                                 1377                                   1404
GCC CTC AAT GCG CAC TGG AGC GCG GAC GCC GTG TAC CAG GAG GCG CGC AAG GTC
Ala Leu Asn Ala His Trp Ser Ala Asp Ala Val Tyr Gln Glu Ala Arg Lys Val

                                 1431                                   1458
GTG GGC GCT CTG CAC CAG ATC ATC ACC CTG AGG GAT TAC ATC CCC AGG ATC CTG
Val Gly Ala Leu His Gln Ile Ile Thr Leu Arg Asp Tyr Ile Pro Arg Ile Leu
```

## FIG. 7C

```
                         1485                                    1512
GGA CCC GAG GCC TTC CAG CAG TAC GTG GGT CCC TAT GAA GGC TAT GAC TCC ACC
Gly Pro Glu Ala Phe Gln Gln Tyr Val Gly Pro Tyr Glu Gly Tyr Asp Ser Thr


                         1539                                    1566
GCC AAC CCC ACT GTG TCC AAC GTG TTC TCC ACA GCC GCC TTC CGC TTC GGC CAT
Ala Asn Pro Thr Val Ser Asn Val Phe Ser Thr Ala Ala Phe Arg Phe Gly His
     ¤


                         1593                                    1620
GCC ACG ATC CAC CCG CTG GTG AGG AGG CTG GAC GCC AGC TTC CAG GAG CAC CCC
Ala Thr Ile His Pro Leu Val Arg Arg Leu Asp Ala Ser Phe Gln Glu His Pro


                         1647                                    1674
GAC CTG CCC GGG CTG TGG CTG CAC CAG GCT TTC TTC AGC CCA TGG ACA TTA CTC
Asp Leu Pro Gly Leu Trp Leu His Gln Ala Phe Phe Ser Pro Trp Thr Leu Leu


                         1701                                    1728
CGT GGA GGT GGT TTG GAC CCA CTA ATA CGA GGC CTT CTT GCA AGA CCA GCC AAA
Arg Gly Gly Gly Leu Asp Pro Leu Ile Arg Gly Leu Leu Ala Arg Pro Ala Lys


                         1755                                    1782
CTG CAG GTG CAG GAT CAG CTG ATG AAC GAG GAG CTG ACG GAA AGG CTC TTT GTG
Leu Gln Val Gln Asp Gln Leu Met Asn Glu Glu Leu Thr Glu Arg Leu Phe Val


                         1809                                    1836
CTG TCC AAT TCC AGC ACC TTG GAT CTG GCG TCC ATC AAC CTG CAG AGG GGC CGG
Leu Ser Asn Ser Ser Thr Leu Asp Leu Ala Ser Ile Asn Leu Gln Arg Gly Arg
           ¤


                         1863                                    1890
GAC CAC GGG CTG CCA GGT TAC AAT GAG TGG AGG GAG TTC TGC GGC CTG CCT CGC
Asp His Gly Leu Pro Gly Tyr Asn Glu Trp Arg Glu Phe Cys Gly Leu Pro Arg


                         1917                                    1944
CTG GAG ACC CCC GCT GAC CTG AGC ACA GCC ATC GCC AGC AGG AGC GTG GCC GAC
Leu Glu Thr Pro Ala Asp Leu Ser Thr Ala Ile Ala Ser Arg Ser Val Ala Asp
```

### FIG. 7D

```
                                 1971                              1998
AAG ATC CTG GAC TTG TAC AAG CAT CCT GAC AAC ATC GAT GTC TGG CTG GGA GGC
Lys Ile Leu Asp Leu Tyr Lys His Pro Asp Asn Ile Asp Val Trp Leu Gly Gly

                                 2025                              2052
TTA GCT GAA AAC TTC CTC CCC AGG GCT CGG ACA GGG CCC CTG TTT GCC TGT CTC
Leu Ala Glu Asn Phe Leu Pro Arg Ala Arg Thr Gly Pro Leu Phe Ala Cys Leu

                                 2079                              2106
ATT GGG AAG CAG ATG AAG GCT CTG CGG GAC GGT GAC TGG TTT TGG TGG GAG AAC
Ile Gly Lys Gln Met Lys Ala Leu Arg Asp Gly Asp Trp Phe Trp Trp Glu Asn

                                 2133                              2160
AGC CAC GTC TTC ACG GAT GCA CAG AGG CGT GAG CTG GAG AAG CAC TCC CTG TCT
Ser His Val Phe Thr Asp Ala Gln Arg Arg Glu Leu Glu Lys His Ser Leu Ser

                                 2187                              2214
CGG GTC ATC TGT GAC AAC ACT GGC CTC ACC AGG GTG CCC ATG GAT GCC TTC CAA
Arg Val Ile Cys Asp Asn Thr Gly Leu Thr Arg Val Pro Met Asp Ala Phe Gln

                                 2241                              2268
GTC GGC AAA TTC CCC GAA GAC TTT GAG TCT TGT GAC AGC ATC ACT GGC ATG AAC
Val Gly Lys Phe Pro Glu Asp Phe Glu Ser Cys Asp Ser Ile Thr Gly Met Asn

                                 2295                              2322
CTG GAG GCC TGG AGG GAA ACC TTT CCT CAA GAC GAC AAG TGT GGC TTC CCA GAG
Leu Glu Ala Trp Arg Glu Thr Phe Pro Gln Asp Asp Lys Cys Gly Phe Pro Glu

                                 2349                              2376
AGC GTG GAG AAT GGG GAC TTT GTG CAC TGT GAG GAG TCT GGG AGG CGC GTG CTG
Ser Val Glu Asn Gly Asp Phe Val His Cys Glu Glu Ser Gly Arg Arg Val Leu

                                 2403                              2430
GTG TAT TCC TGC CGG CAC GGG TAT GAG CTC CAA GGC CGG GAG CAG CTC ACT TGC
Val Tyr Ser Cys Arg His Gly Tyr Glu Leu Gln Gly Arg Glu Gln Leu Thr Cys

                                 2457                              2484
ACC CAG GAA GGA TGG GAT TTC CAG CCT CCC CTC TGC AAA GAT GTG AAC GAG TGT
Thr Gln Glu Gly Trp Asp Phe Gln Pro Pro Leu Cys Lys Asp Val Asn Glu Cys
```

## FIG. 7E

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```
                     2511                                  2538
GCA GAC GGT GCC CAC CCC CCC TGC CAC GCC TGT GCG AGG TGC AGA AAC ACC AAA
Ala Asp Gly Ala His Pro Pro Cys His Ala Ser Ala Arg Cys Arg Asn Thr Lys

                     2565                                  2592
GGC GGC TTC CAG TGT CTC TGC GCG GAC CCC TAC GAG TTA GGA GAC GAT GGG AGA
Gly Gly Phe Gln Cys Leu Cys Ala Asp Pro Tyr Glu Leu Gly Asp Asp Gly Arg

                     2619                                  2646
ACC TGC GTA GAC TCC GGG AGG CTC CCT CGG GTG ACT TGG ATC TCC ATG TCG CTG
Thr Cys Val Asp Ser Gly Arg Leu Pro Arg Val Thr Trp Ile Ser Met Ser Leu

                     2673                                  2700
GCT GCT CTG CTG ATC GGA GGC TTC GCA GGT CTC ACC TCG ACG GTG ATT TGC AGG
Ala Ala Leu Leu Ile Gly Gly Phe Ala Gly Leu Thr Ser Thr Val Ile Cys Arg

                     2727                                  2754
TGG ACA CGC ACT GGC ACT AAA TCC ACA CTG CCC ATC TCG GAG ACA GGC GGA GGA
Trp Thr Arg Thr Gly Thr Lys Ser Thr Leu Pro Ile Ser Glu Thr Gly Gly Gly

                     2781                                  2808
ACT CCC GAG CTG AGA TGC GGA AAG CAC CAG GCC GTA GGG ACC TCA CCG CAG CGG
Thr Pro Glu Leu Arg Cys Gly Lys His Gln Ala Val Gly Thr Ser Pro Gln Arg

                     2835                                  2862
GCC GCA GCT CAG GAC TCG GAG CAG GAG AGT GCT GGG ATG GAA GGC CGG GAT ACT
Ala Ala Ala Gln Asp Ser Glu Gln Glu Ser Ala Gly Met Glu Gly Arg Asp Thr

                     2889                                  2916
CAC AGG CTG CCG AGA GCC CTC TGA GGG CAA AGT GGC AGG ACA CTG CAG AAC AGC
His Arg Leu Pro Arg Ala Leu ^^^

                     2943                                  2970
TTC ATG TTC CCA AAA TCA CCG TAC GAC TCT TTT CCA AAC ACA GGC AAA TCG GAA

                     2997                                  3024
ATC AGC AGG ACG ACT GTT TTC CCA ACA CGG GTA AAT CTA GTA CCA TGT CGT AGT

                     3051
TAC TCT CAG GCA TGG ATG AAT AAA TGT TAT AGC TGC AAA AAA AAA AAA
                      ^^^ ^^^
```

## FIG. 7F

FIG. 9 SUBSTITUTE SHEET

FIG. 10A



FIG. 10B

HUMAN THYROID
MICROSOMES



r = 0.902

CHO - HTPO MICROSOMES

*F I G. 10C*

OPTICAL
DENSITY



● SERUM 11
■ SERUM 27
○ SERUM 12
□ SERUM 28
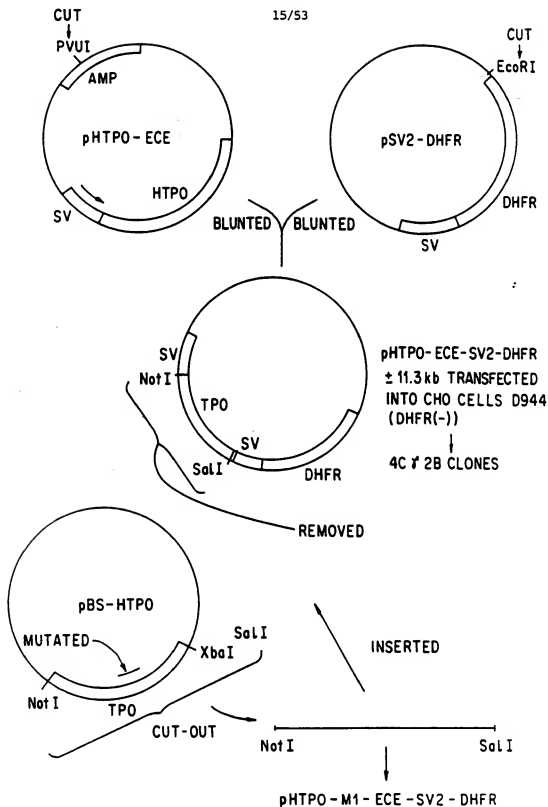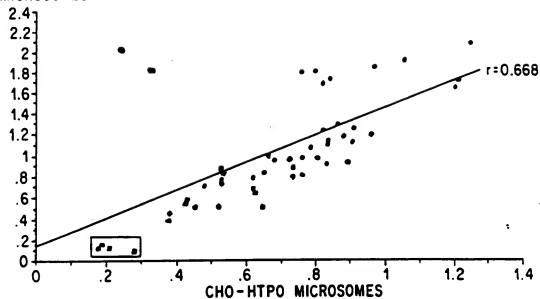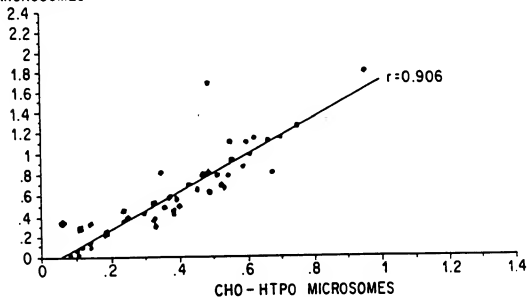
LOG SERUM DILUTION
HUMAN THYROID MICROSOMES

*F I G. 11A*

SUBSTITUTE SHEET
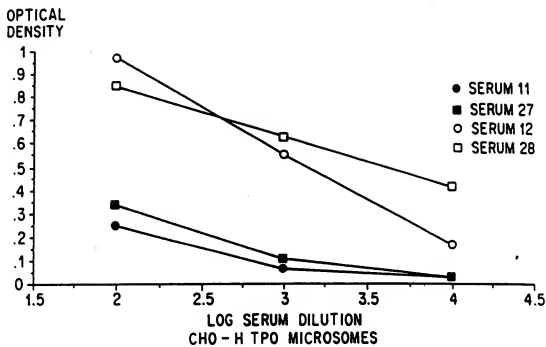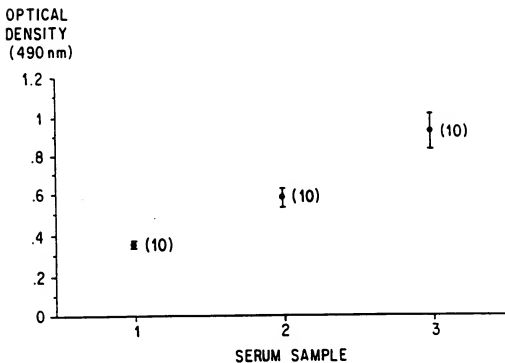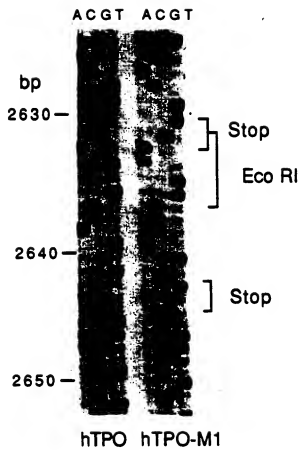
OPTICAL
DENSITY

● SERUM 11
■ SERUM 27
○ SERUM 12
□ SERUM 28

LOG SERUM DILUTION
CHO – H TPO MICROSOMES

*F I G. 11B*



OPTICAL
DENSITY
(490 nm)

SERUM SAMPLE

*F I G. 12*

FIG. 13

FIG. 14A

F I G. 14B

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| HOURS of CHASE | 0 4 8 12 24 | 0 4 8 12 24 |
| --- | --- | --- |
| | CELLS | MEDIUM |
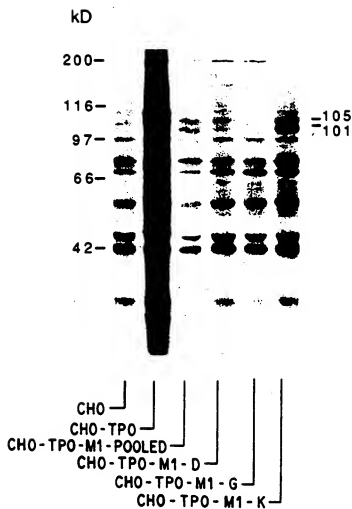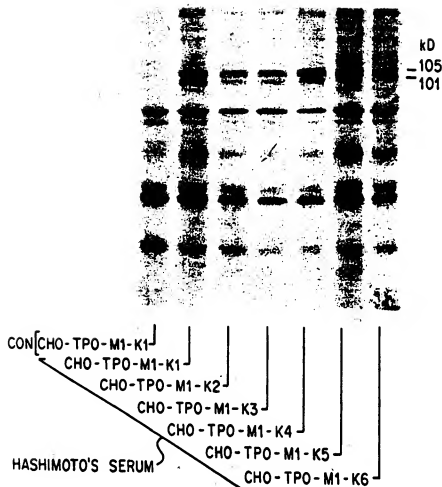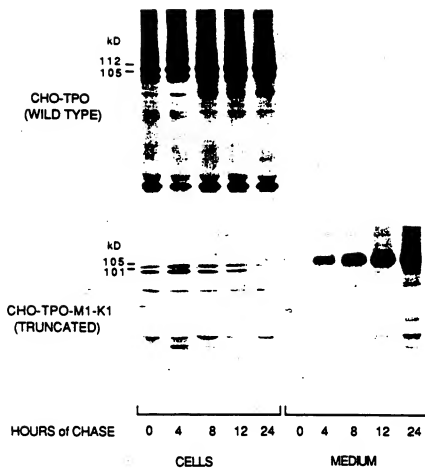
*F I G. 15*

SUBSTITUTE SHEET

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*F I G. 16*
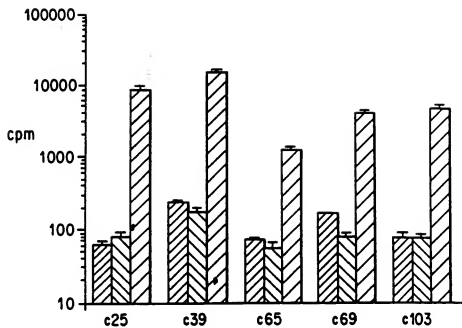
*F I G. 17A*



*F I G. 17B*

**SUBSTITUTE SHEET**

| CLONE | hTPO bp | | |
|-------|---------|---|---|
| E | 782 | | 1123 |
| F | 800 | 990 | |
| M | 833 | 1068 | |
| N | 881 | 1032 | |
| O | 854 | 1077 | |
| P | 818 | 939 | |
| Q | 851 | 1095 | |
| R | 845 | 1086 | |
| S | 878 | 1107 | |
| T | 764 | 936 | |
| U | 746 | 960 | |
| V | 767 | 927 | |
| Y | 837 | 1011 | |
| Z | 851 | 1150 | |
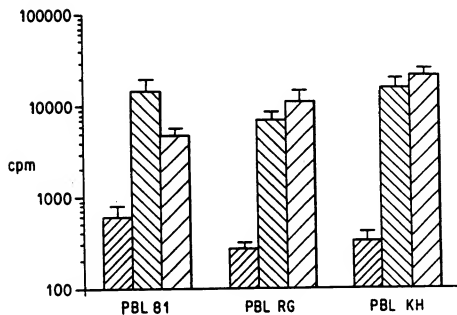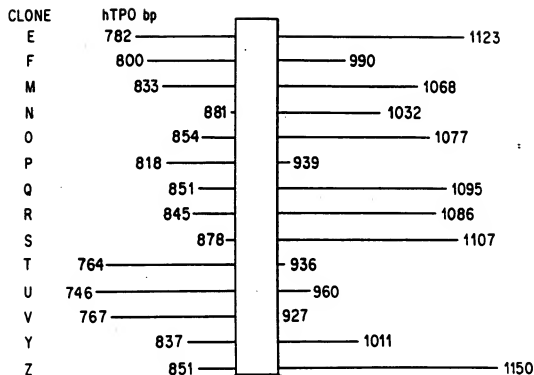
NUCLEOTIDE
883 AAC CCA TGT TTT CCC ATA CAA CTC CCG GAG GAG GCC CGG CCG GCC 927
AMINO ACID
266 Asn Pro Cys Phe Pro Ile Gln Leu Pro Glu Glu Ala Arg Pro Ala 281

*FIG. 18*

FIG.19

FIG. 20
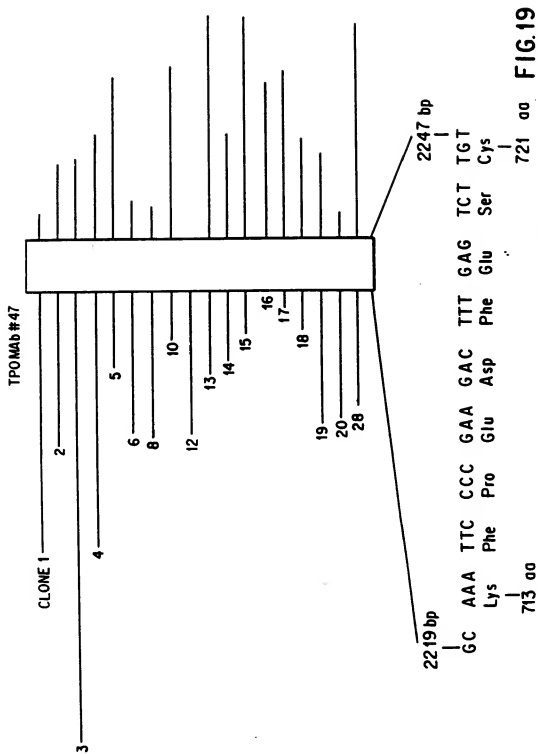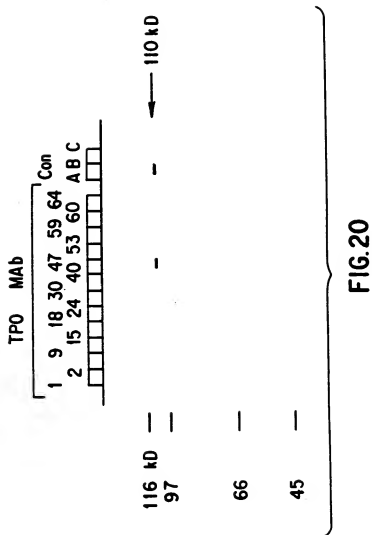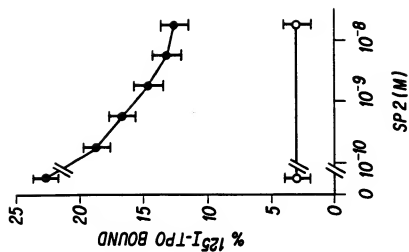
FIG. 28
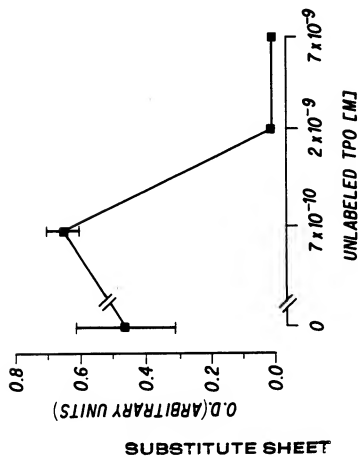
FIG. 21

## VH SEGMENT (VHI)

```
        Q   V   K   L   L   E   S   G   A   E   V   K   K   P   G   A   S   V   K   V
SP2   CAGGTGAAACTGCTCGAGTCTGGGGCTGAGGTGAAGAAGCCTGGGGCCTCAGTGAAGGTC      60
1-1   ----CAG---G-GC---C--------------------------T---------------
                                        ********** CDR1

        S   C   K   A   S   G   Y   T   F   T   G   H   Y   M   H   W   V   R   Q   A
SP2   TCCTGCAAGGCTTCTGGATACACCTTCACGGGCCACTATATGCACTGGGTGCGACAGGCC    120
1-1   --------------------------T--------------------------------
                                        *********** CDR2

        P   G   Q   G   L   E   W   I   G   W   I   S   P   N   R   G   A   T   R   F
SP2   CCTGGACAGGGGCTTGAGTGGATAGGATGGATCAGCCCTAACAGGTGCCACAAGGTTT    180
1-1   ----------------------G--------A-----T---G---C-AC-A-

        A   Q   K   F   Q   G   R   V   T   M   T   S   D   T   S   I   N   T   V   Y
SP2   GCACAGAAGTTTCAGGGCAGGGTCACCATGACCAGCGACACGTCCATTAACACAGTCTAC    240
1-1   *********--------------------------G-----C-G---C-G-----C
      *********

        M   E   L   S   G   L   R   F   D   D   T   A   V   Y   Y   C   A   T
SP2   ATGGAGCTGAGCGGCCCTGAGATTGACGACACAGCGGGCGTGTATTACTGTGCGACA
1-1   ---------------AAG---------C---------------------G--
```

FIG. 22

D SEGMENT    CDR3

```
                     * * * * * * * * * * * * * * * * * * * *
                     T  R  T  A  Y  Y  G  M  D
              SP2   ACACGCACGGGCCTACTACGGTATGGAC
```

JH SEGMENT (JH3)

```
                      R  L  G  P  R  D  H  G  H  R  L  F
              SP2   A·G·······GTCTGGGGGCCAAGGGACCACGGTCACCGTCTCTCA
              JH3   ATGCTTTTGAT--------------------A-T------------------
```

FIG. 22 (CONT.)

## VL SEGMENT (VKI)

```
             E   L   V   M   T   Q   S   P   S   S   L   S   A   S   E   G   D   T   V   T
SP2     GAGCTCGTGATGACCCAGTCTCCATCTCCCTGTCTGCATCTGAGGGAGACACAGTCACC                      60
KLVJ    --CA--CA-----------------------C--------------------TA----------G---------I-----

                                     CDR1
                                     ****** ******* ****
             I   T   C   R   A   S   E   N   I   S   R   Y   S   N   W   Y   Q   Q   Q   P
SP2     ATCACTTGCCGGGCAAGTGAGAATATTAGCAGGTATTCAAATTGGTATCAGCAGCAACCA                      120
KLVJ    ----------------------------C---GC---T-AC---T------------A---

                                           CDR2
                                           ********* ***********
             G   K   A   P   K   L   L   I   S   A   A   S   T   L   Q   S   G   V   P   S
SP2     GGGAAAGCCCCTAAACTCCTGATCTCTGCTGCATCCACTTTACAAAGTGGGGTCCCATCA                       180
KLVJ    ------------G------------------A------------G--G--------G----

             R   F   S   G   S   G   S   G   T   H   F   T   L   T   I   H   N   S   L   Q   P
SP2     AGGTTCAGTGGCAGTGGATCTGGGACACATTTCACTCTCACCATCCACAGTCTGCAACCT                       240
KLVJ    ------------------------------G---------------------G-------

                                 CDR3
                                 ******** ***************
             G   D   F   A   T   Y   Y   C   Q   Q   T   Y   S   S   P   F
SP2     GGAGATTTTGCAACTTACTACTGTCAACAGACTTACAGTCCCCGTTC
KLVJ    -A----------------------------G------------A----ACC-T
```

## J SEGMENT (JK2)

```
             T   F   G   Q   G   T   K   L   E   I   K   R   T
SP2     ACTTTTGGCCAGGGGACCAAGCTGGAGATCAAACGAACT
KV312   ----------------------------------------
```

## FIG. 23

FIG. 24

SUBSTITUTE SHEET

```
KLVJ       G......G.. .G.........  ..................  ..........T..  ..........A..    200
VK-8P2     G......C.. .A.........  ..................  ..........T..  ..........A..    200
VK-8P4     G......C.. .G.........  ..................  ..........G..  ..........C..    200
VK-8P5     A......C.. .G.........  ..................  ..........T..  ..........G..    200

Consensus  -CATCCA-TT T-CAAAGTGG GGTCCCATCA AGGTTCAG-G GCAGTGG-TC    200
                      **************
                         CDR2

KLVJ       ..........  ..........G.  ..................  .A..........  250
VK-8P2     ..........  ..........C.  ..................  .G..........  250
VK-8P4     ..........  ..........G.  ..................  .A..........  250
VK-8P5     ..........  ..........G.  ..................  .A..........  250

Consensus  TGGGACA-AT TTCACTCTCA CCATCA-CAG TCTGCAACCT G-AGATTTTG    250

KLVJ       ..........  ..........  .G......A ...ACCT      288
VK-8P2     ..........  ..........  .C.......T ...CGTT      288
VK-8P4     ..........  ..........  .G.......A ...CGTG      288
VK-8P5     ..........  ..........  .G.......A ...CGTG      288

Consensus  CAACTTACTA CTGTCAACAG A-TTACAGT- CCC----     288
                      ********************
                           CDR3
```

FIG. 24 (CONT.)

```
                                                         50
KLVJ       DI................................S.S N................A    50
VK-SP2     ..........B..T.......BN.S R.S.....Q........SA               50
VK-SP4     .........................TVG T..............B.....T         50
VK-SP5     .........S......N.G K.....R...........B....G                50

Consensus  ELVMTQSPSS LSASVGDRVT ITCRASQ.I. .YLNWYQQKP GKAPKLLIY.      50
                                 ********    ********
                                  CDRJ        *

                                                         96
KLVJ       .S..................................G.........T..S.V       96
VK-SP2     ...............H.............N..................V           96
VK-SP4     ...........R...............................V               96
VK-SP5     T..........................................V               96

Consensus  ASTLQSGVPS RFSGSGSGTD FTLTISSLQP EDFATYYCQQ SYSTPP         96
           *****                                      *******
           CDR2                                        CDR3
```

## FIG. 25

```
J-KV312    ..T..T.... .G........ .C....G... .........   39
JK-SP2     ..T..T.... .G........ .C....G... .........   39
JK-SP4/5   ..G..C.... .A........ .G....A... .........   39
Consensus  AC-TT-GGCC A-GGACCAA  G-TGGA-ATC AAACGAACT    39


J-KV312    .......... ...   13
JK-SP2     .......... ...   13
JK-SP4/5   ......V.. ...   13
Consensus  TFGQGTKLEI KRT   13
```
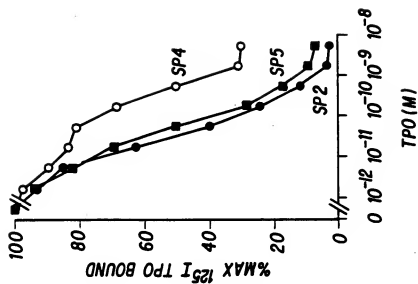
FIG. 26

FIG. 27B



FIG. 27A

FIG. 29B



FIG. 29A

FIG. 30B



FIG. 30A

```
                                                            CDR1
Consensus  CAGGTG-A-C TG-T--AGTC TGGGGCTGAG -TGAA-AA-C CTGGGGCCTC   50
hv1L1      ......C.G. ..G.GC.... .......... G....C..G. ..........   50
SP4.6      ......A.A. ..C.CG.... .......... T....G..C. ..........   50

Consensus  AGTGA-GGTC TCCTGCAAGG CTTCTGGA-A CACCTTCA-C G-CTAC-AT-   00
hv1L1      ......A... .......... .......G.. .........C .G....T..A   00
SP4.6      ......G... .......... .......T.. .........A .A...C..G   00

Consensus  T-CACTGGGT GCGACAGGCC CCTGGACAAG GGCTTGAGTG G-TGGGA TGG  150
hv1L1      .G........ .......... .......... .......... .A.......  150
SP4.6      .T........ .......... .......... .......... .G.......  150
```

FIG. 31A
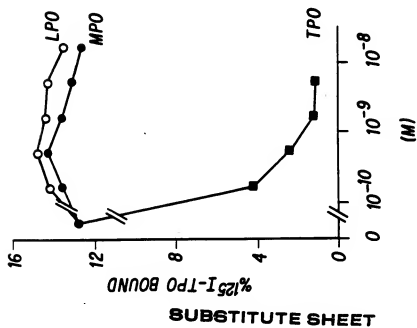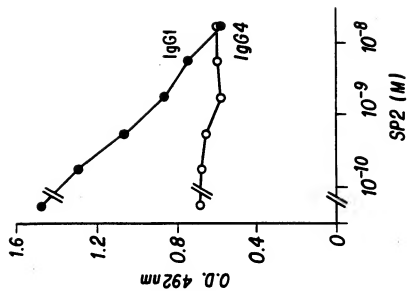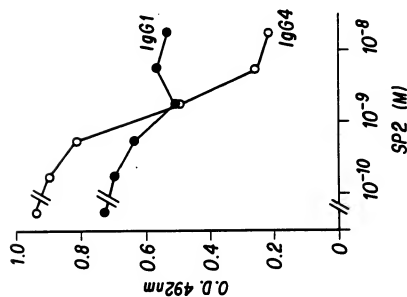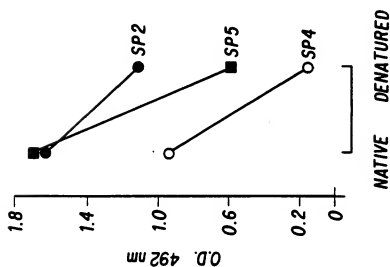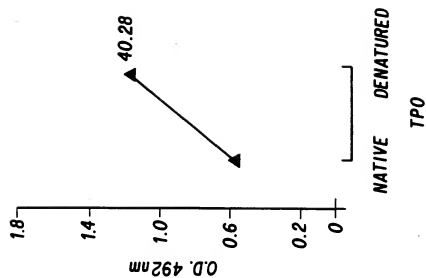
CDR2

```
                                                    ┌─────────────────────────────┐
Consensus   ATCAACCCTA  A-A-TG-TGG  CAC-A--TAT  C-CAGGAGT  TTCAGGGCAG           200
hv1L1       ..........  .C.G..G...  ...A.AC...  G.A......  ..........           200
SP4.6       ..........  .G.A..C...  ...G.GA...  T.C......  ..........           200
                                                    └─────────────────────────────┘

Consensus   GGTCACCATG  ACCAG-GACA  CG--CCATCAG  C-CAGCCTA-  ATGGAGCTGA          250
hv1L1       ..........  .....G....  ..T......  .A........C  ..........          250
SP4.6       ..........  .....A....  ..G......  .G........T  ..........          250

Consensus   -CAG-CTGA-  ATC-GACGAC  ACGGCCGT-T  A-TACTGTGC  G-GA                294
hv1L1       G...G....G  ...T......  .........G  .T........  .A..                294
SP4.6       C....C....A ...G......  .........C  .C........  .C..                294
```

FIG. 31A (CONT.)

```
                       CDR1
                       ┌──────────────┐
Consensus  QV-L-SGAE ---PGASV-V SCKASG-TF- -Y--HWVRQA PGQGLEW-GW  50
hv1L1      ..Q.VQ.... VNK.....K. ......D..T  G.YM..... ......M.  50
SP4.6      ..K.LE.... LKN.....R. ......Y..N  D.HV..... ......V.  50

           CDR2
           ┌──────────┐
Consensus  INP---GT-Y -QKPQGRVTM TRDT-IS-AY MEL--L-SDD TAVYYCAR  98
hv1L1      ...NSG.N. A. ......S.T. ...SR.R... ........  98
SP4.6      ...KNA.R. S. ......A..A. ...TS.K... ........  98
```

FIG. 31B

```
SP4.6  GGG GTA GGA GTT GGT ACG TGG GGC CTT
        G   V   G   V   G   T   W   G   L
```

FIG. 31C

SUBSTITUTE SHEET

Consensus    EIVMTQSPSS LSASVGDRVT ITC`RASQ-IS -YLNWYQQKP` GRAPKLLIY-    A    50

CDR1

KJVJ          DI.......                                                        50
SP # 14,15,21,22  ........                  .S.. .S. N..    .....  .....  .B.   G  50
18            ........   .....   ....  .N.G K..   R...   ..N..  .     50
20            ........   ...B.   ....  .RA. T..            R...N.  B   50
12            ........   ...G.   ....  ..S. A..   .K...         .    50
13            ........            ....  T.T. R..   ..I       .FA.  50
16            ........   .....   ....  ..D. R..           .H.  50
17,19         ........   ..B.T.  ....  .EN. R.S   .Q.    .S.  50

CDR2

Consensus    ASTLQS`GVPS RFSGSGSGTD FTLTISSLQP EDFATYYCQQ` SYSTP-    96

CDR3

KJVJ                                                        HL   96
SP # 14,15,21,22  T.  .S.                                .S.Y  96
18            T..                      .F..            .S.Y  96
20            .S.                      .F..            .D.W  96
12            .S.                .T..            .D.VD  96
13            .T.   ....G....          .D...           .T.Y  96
16            .B.   ...G...                       .Y...  .F  96
17,19         ...   ....H...     .N....      .G...   T.S.F  96

FIG. 32A

FIG. 32B

FIG. 33

FIG. 29C

FIG. 34

SUBSTITUTE SHEET

FR3     CDR3

```
                     QGRVTMTRDTSISTAYMELSRLRSDDTAV  YYCAR  GLGVGTWGL         JH4
EV1L1         .......A..AT....TS..A....         .F...  .........
WR4.10        .......A..AT....TS..A....
WR4.12        .......A..AT....TS..A....
WR4.28        .......A..AT....TS..A....         .F...  ....I....

WR4.27        .......A..AV....TS..A....         .F...
WR4.34        .......A..AV....TS..A....
WR4.35        .......A..AV....TS..A....
WR4.32        .......A..AV....TS..A....
WR4.31        .......A..AV....TS..A....         .H...  .VP.....
WR4.25        ....AG.G..A..AT....TS..A....       .F...  .V......
JP4.6         ...........A..A..VTS.....          .....  .V......
JA1.3         ....S....A..A...TS.K....           .....  TRTA....
SP1.2         ....S....N.V....G..F....           .F...  .........

8-1B          GRFTISRDNSKNTLYLQMNSLRAEDTAV       YYCAR  TQGTRSYY           JH4
TR1.3         ...L......E..V.........K           .....K S.......
TR1.5         ...........D..I.......T.D...L       .....K

V35           QGRVTSTRDTSISTAYMELSRLRSDDTVV      YYCAR  DFYGGGKSE          JH4
TR1.9         R...F......AT...G..S..PE..A.        .....

EV1263        QGRVTTADKSTSTAYMELSSLRSEDTAV       YYCA   GTWGDPLPLSV        JH3
TR1.6         ...S..........NI...D.NT.T....A.I    ....T  ANDRGAYASFG
TR1.8         ...K......S......NI...D.NT.T....A.I

3-1           QGRVTMTRDTSTVYMELSLRSEDTAV         YYCA   RGDSNIWTLG         JH4
WR1.7         ...I....A..S......T....            .....S VLGIIADH
WR1.9         ...I....A..S......T....            .....R VLGIIADH
TR1.10        ...I....A..L.MN....                .....R
```

JH6 .

JH6 .

FIG. 34 (CONT.)

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FIG. 35

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**FIG. 35 (CONT.)**

FR3    CDR3

KL012
TRI.10
JA.9
SP1.4
TRI.5
SP1.5
WR4.7
WR1.2
WR1.35
WR1.33
WR1.12
WR1.45
WR1.39
WR1.37
WR1.32
WR1.22
WR1.8
WR1.6
WR1.21
WR1.10
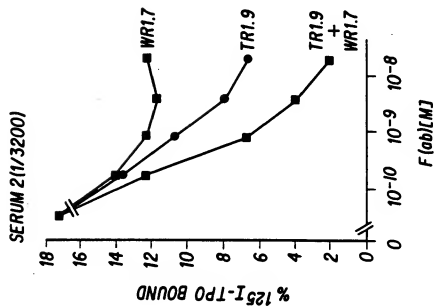SP1.12

A'
TRI.13
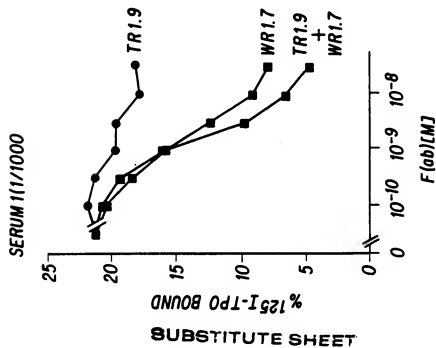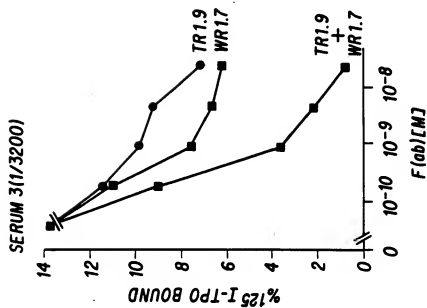TRI.9

A3
TRI.6
TRI.8

FIG. 36B



FIG. 36A

FIG. 36D

FIG. 36C

FIG. 37

FIG. 38B



FIG. 38A

SUBSTITUTE SHEET

FIG. 38C